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**THE UNIVERSITY OF EDINBURGH**

**THE EFFECT OF ENDOTHELIN A AND ENDOTHELIN B RECEPTOR  
LIGANDS ON THE CARDIOVASCULAR SYSTEM OF MAN**

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## ABSTRACT

**Background:** The endothelin (ET) system, implicated in the pathogenesis of several cardiovascular diseases, acts through two receptors; ET<sub>A</sub> and ET<sub>B</sub>. Both are present on myocardial cells and vascular smooth muscle cells mediating vasoconstriction while the ET<sub>B</sub> is also present on endothelial cells mediating vasodilatation. Thus, the cardiovascular effects of ET will depend, in part, on the balance of action at these 2 receptors. In addition, the ET<sub>B</sub> receptor may also act as a clearance receptor. ET receptor antagonists (ETRA<sub>s</sub>) are being developed as clinical therapies. The role of the ET<sub>B</sub> receptor may determine which will be most beneficial. Recently, a novel form of ET has been described; ET<sub>[1-31]</sub>. It is unclear whether this is vasoactive in its own right, or is further processed to ET-1 suggesting a novel pathway of ET-1 production.

**Aims:** To investigate: 1. The vascular effects of ET<sub>[1-31]</sub>, and whether there are increased plasma concentrations in patients with chronic heart failure (CHF). 2. The effects of ET-1 and its antagonists on isolated human myocardium and its interaction with the beta-adrenergic system. 3. The role of the ET<sub>B</sub> receptor in CHF and whether selective ET<sub>A</sub>RA have different haemodynamic effects than dual ET<sub>A/B</sub>RA. 4. The role of the ET<sub>B</sub> in conditions of endothelial dysfunction (hypercholesterolaemia) and whether any dysfunction is reversible with 'statin' therapy.

**Methods:** Skin blood flow after intra-dermal injection of ET ligands was developed and assessed using laser Doppler flowmetry. Forearm blood flow after intra-arterial infusion of ET ligands was measured by venous occlusion plethysmography. Large artery stiffness was assessed by pulse wave analysis. Systemic haemodynamics were determined invasively by the thermodilution method and non-invasively by thoracic bioimpedance. Direct ET effects on human myocardium were developed and assessed in isolated human right atrial trabeculae. The effects of lowering plasma cholesterol was assessed following 8 weeks of 'statin' therapy. Plasma ET concentrations were determined using immunoassays.

**Results:** ET-1 vasoconstricts in the skin microcirculation and it appears that endothelin converting enzyme (ECE) activity is present as big ET-1 also vasoconstricts. ECE and neutral endopeptidase (NEP) blockade both cause vasodilatation suggesting skin basal resting ET tone. ET-1<sub>[1-31]</sub> is also a vasoconstrictor in the skin microcirculation. Plasma concentrations of ET-1<sub>[1-31]</sub> are not elevated in patients with CHF. ET<sub>A</sub>RA improves systemic haemodynamics in patients with CHF while concomitant ET<sub>B</sub>RA attenuates this effect. In the isolated human myocardium ET is positively inotropic but there is no resting ET inotropy with no effect on basal twitch force with ETRA. In addition, ET attenuates beta-adrenergic activation in isolated human myocardium. In hypercholesterolaemia, forearm vascular effects are similar to those previously reported in healthy volunteers. Treatment with statin therapy for 8 weeks caused a trend towards an increase in ET<sub>A</sub> mediated vasodilatation.

**Conclusions:** The novel finding that ET<sub>[1-31]</sub> is a vasoconstrictor in the skin microcirculation may represent a novel pathway of ET production. The haemodynamic benefits of selective ET<sub>A</sub>RA over dual ET<sub>A/B</sub>RA is a unique finding with considerable importance and supports the development of selective ET<sub>A</sub>RA<sub>s</sub> as clinical therapies in CHF. The finding of antagonism between the ET system and the beta-adrenergic stimulation may represent a protective adaptation in conditions where there beta-adrenergic stimulation is detrimental and there is activation of the ET system, such as CHF.

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## **DECLARATION**

This thesis represents work performed in the Clinical Pharmacology Unit and Research Centre and Department of Cardiology at the Western General Hospital, Edinburgh between 1998 and 2001 while I was a British Heart Foundation Junior Research Fellow. The vast majority of the work described here has been my own. Other researchers involved have been acknowledged. Sections of the work have been published in peer reviewed journals and this has been acknowledged in the text and Bibliography. The thesis has not been accepted in any previous application for a degree and all sources of information have been acknowledged.

A handwritten signature in black ink, appearing to read 'S. J. Leslie', with a stylized, flowing script.

Stephen James Leslie

**July 2004**

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Finally, I would like to thank my parents for encouraging me to ask 'but why?'. I dedicate this thesis to them.

## ABBREVIATIONS

ACE	Angiotensin converting enzyme
ANOVA	Analysis of variance
AUC	Area under the curve
Big ET-1	Endothelin -1 [1-38]
CAD	Coronary artery disease
CCO	Continuous cardiac output
DBP	Diastolic blood pressure
ECE	Endothelin converting enzyme
ET <sub>A</sub>	Endothelin receptor - type A
ET <sub>B</sub>	Endothelin receptor - type B
ET-1 <sub>[1-21]</sub>	Endothelin -1 [1-21]
ETRA	Endothelin receptor antagonist
ETR	Endothelin receptor
ET-1 <sub>[1-31]</sub>	Endothelin -1 [1-31]
F2001	Filtrass 2001
FBF	Forearm blood flow
HEC4	Hokanson EC4
HR	Heart rate
LBNP	Lower body negative pressure
MAP	Mean arterial pressure
MPAP	Mean pulmonary arterial pressure
NEP	Neutral Endopeptidase
PCWP	Pulmonary capillary wedge pressure
PVR	Pulmonary vascular resistance
SBF	Skin blood flow
SBP	Systolic blood pressure
SCWT	Stroop's coloured word test
SEM	Standard error of the mean
SD	Standard deviation
SVR	Systemic vascular resistance

## **Chapter 1**

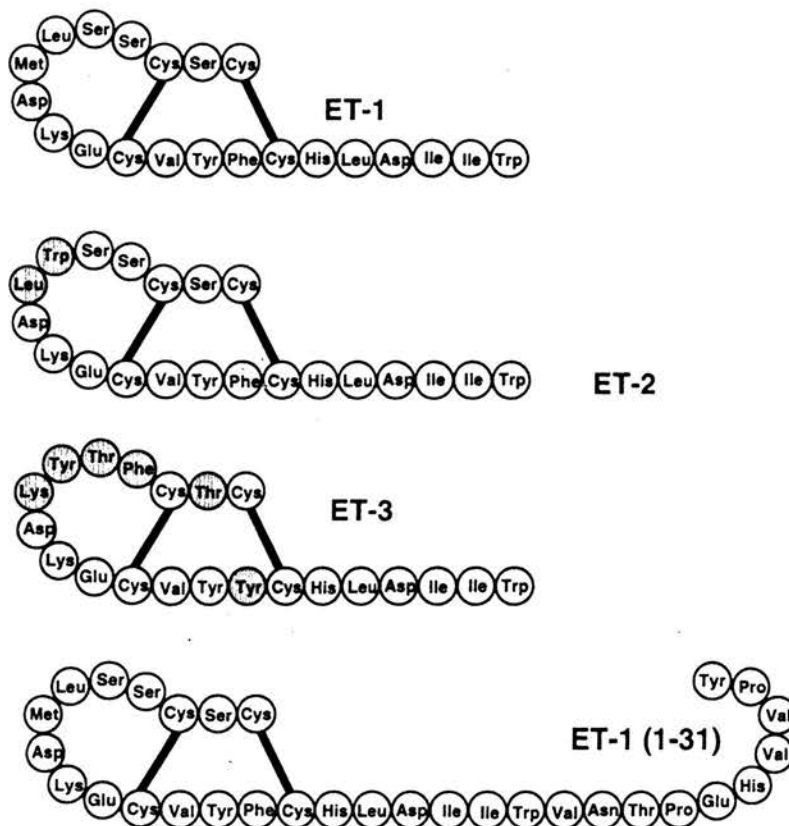
### **Introduction - the endothelin system**

**Leslie SJ**, Webb DJ. Endothelin and its Inhibitors. In: Endothelin Ligands and their Experimental Effects within the Human Circulation. *Handbook of Physiology*. Springer 2001 Chapter 19.

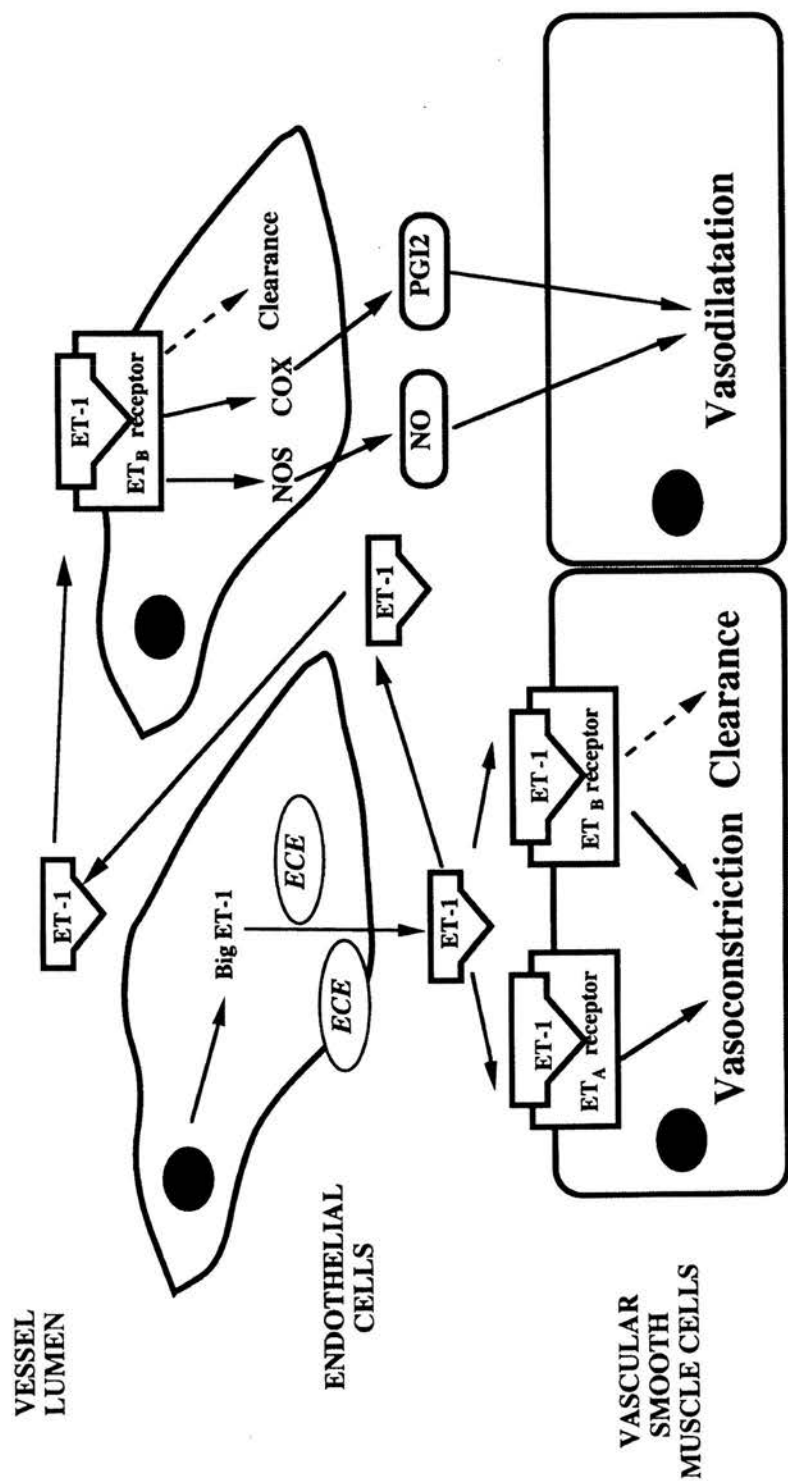
## 1.1 THE ENDOTHELIN SYSTEM

Endothelin (ET) was discovered in 1988 [Yanagisawa *et al* 1988]. It is a 21 amino-acid polypeptide with two disulphide bonds which has three isoforms, ET-1, ET-2 and ET-3 [Inoue *et al* 1989] (Figure 1.1). Two receptor subtypes have been identified ET<sub>A</sub> and ET<sub>B</sub> (Figure 1.2). ET-1 and ET-2 have a higher affinity than ET-3 for the ET<sub>A</sub> receptor while all three have a similar affinity for the ET<sub>B</sub> receptor. The different isoforms are produced by a variety of cells but the most important isoform, predominantly produced by the endothelial cell, is ET-1. ET-1 is the most potent vasoconstrictor yet discovered in humans. In addition, it has mitogenic properties and is positively inotropic [Meyer *et al* 1996]. A substantial body of evidence has developed implicating the ET system in basal vascular tone and the pathogenesis of several cardiovascular conditions in humans including, hypertension [Haynes *et al* 1994a], renal failure [Bussemaker *et al* 1998, Ferro *et al* 1998], pulmonary hypertension [McCulloch *et al* 1995 & 1998] and chronic heart failure (CHF) [Cavero *et al* 1990]. Endothelin receptor antagonists (ETRAs) and converting enzyme inhibitors have been developed and many ETRAs are now under development as potential therapies for a variety of conditions [Douglas *et al* 1997]. However, despite this intensive research effort, several important questions remain unanswered.





**Figure 1.1** Diagram of endothelin isoforms, illustrating amino acid sequence and presence of 2 disulphide bonds. The shaded circles denote differences in amino acid structure of ET-2 and ET -3 compared with ET -1.



**Figure 1.2** Schematic of vascular ET<sub>A</sub> and ET<sub>B</sub> receptors (Adapted from Strachan)

## **1.2 ENDOGENOUS SYNTHESIS OF ENDOTHELIN**

### **1.2.1 Endothelin gene product**

The production of ET-1 is regulated at the level of the gene product located on chromosome 6 by many factors (Table 1.1). After transcription and removal of the signal peptide a 212 amino acid peptide, preproendothelin-1 is produced (Figure 1.3). Prepro-ET-1 mRNA is found in the highest concentrations in lung although it can be found in most organs including the heart, kidney, brain, pancreas and spleen. In general, basal physiological production of ET-1 is low but it is inducible [Redington *et al* 1997, Woods *et al* 1999]. In disease conditions, such as atherosclerotic plaques, basal ET-1 production from endothelial cells is increased and this may have important clinical consequences, especially in vascular beds in the heart, brain and kidneys. Preproendothelin-1 is subsequently processed by several enzymes to a 38 amino acid derivative, big ET-1 [Yanagisawa *et al* 1988].

### **1.2.2 Big endothelin-1**

Big ET-1, the 38 amino acid precursor to ET-1, is relatively inactive compared with ET-1 [Kashiwabara *et al* 1989, Hirata *et al* 1990], with a receptor affinity at the ET<sub>A</sub> receptor ~1000 times less than ET-1 [Gray *et al* 1996]. However, when injected intravenously it has similar vascular effects to ET-1 suggesting prompt vascular conversion [Gardiner *et al* 1993]. This conversion is mediated by endothelin converting enzyme but there are several other related proteases which can also cleave big ET-1 (Table 1.2).

## PROMOTORS

### Hormones

- Angiotensin II
- Vasopressin
- Adrenaline
- Insulin
- Cortisol

### Blood Components

- Thrombin
- Activated platelets (lysophosphatidic acid)
- Glucose
- Oxidised Low Density Lipoprotein

### Peptides

- Endothelin
- Endotoxin
- TGF beta
- TNF alpha
- Interleukin – 1
- Cytokines

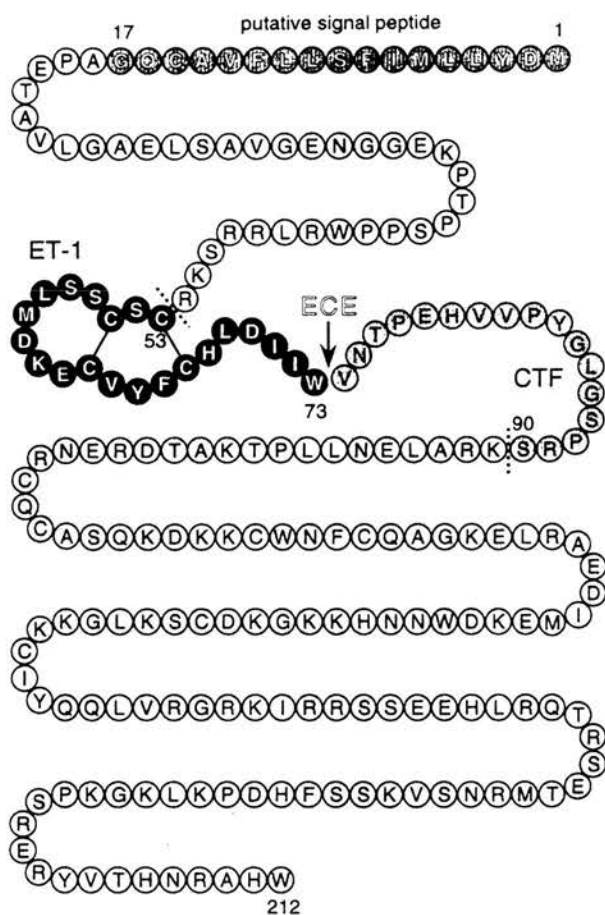
### Others

- Cyclosporin
- Hypoxia
- Low shear stress
- Osmolarity

## INHIBITORS

- Shear stress
- Prostacyclin
- Nitric Oxide
- Natriuretic peptides (ANP, BNP and CNP)

**Table 1.1** Factors influencing endothelin gene product synthesis. (ANP - atrial natriuretic peptide, BNP – brain natriuretic peptide, CNP – c-type natriuretic peptide, TGF – transforming growth factor, TNF – tumour necrosis factor)



**Figure 1.3** Amino acid sequence of preproendothelin and putative signal sequence illustrating cleavage point for endothelin converting enzyme (Adapted from Endothelin Ligands and their Experimental Effects Within the Human Circulation. *Handbook of Physiology*. Springer 2001)

### 1.2.3 Endothelin converting enzymes

There are at least 25 proteases which can convert ET precursors into active ETs (Table 1.2). However, the 'endothelin converting enzymes' (ECEs) [Shimada *et al* 1994, Xu *et al* 1994] are thought to be the most important. ECE-1 consists of at least 4 isoforms although their exact specificities for big ET-1, big ET-2 and big ET-3 are not known. The 4 isoforms currently characterized are ECE-1a, ECE-1b, ECE-1c and ECE-1d and are produced from the same gene products with differences in exon splicing of the 5' region [Valdenaire *et al* 1995 & 1999, Schweizer *et al* 1997]. ECE-2 has similarities with ECE-1 with 59% homology [Emoto & Yanagisawa 1995]. There are several other related peptides, including human mast cell chymase (see below), although their exact number and whether they are physiologically important in ET-1 production is, as yet, unclear (Table 1.2).

### 1.2.4 Endothelin-1

Endothelin-1 is a potent vasoconstrictor agent released most importantly by endothelial cells but also by other cell types including myocytes and fibroblasts. Due to its vasoconstrictor actions, ET-1 has been implicated in a number of cardiovascular diseases including chronic heart failure, hypertension and pulmonary hypertension (see below). The actions of ET-1 are mediated through 2 receptor subtypes in humans; the ET<sub>A</sub> and the ET<sub>B</sub>. ET-1 causes vasoconstriction via the ET<sub>A</sub> and ET<sub>B</sub> receptors located on vascular smooth muscle cells and a prostanoid and nitric oxide (NO) mediated vasodilatation via the ET<sub>B</sub> receptor on endothelial cells. Thus, ET<sub>A</sub> blockade causes

vasodilatation while the effects of ET<sub>B</sub> blockade will depend, in part, on the balance between the vasodilating and vasoconstricting effects at different sites. In healthy volunteers this balance appears to be in favour of vasodilatation as ET<sub>B</sub> receptor blockade causes vasoconstriction (see below).

### 1.2.5 Endothelin-1<sub>[1-31]</sub>

Recently, new derivatives of big ETs have been characterised in humans; ET-1<sub>[1-31]</sub> is generated following the cleavage of big ET-1 at the Tyr<sup>31</sup>–Gly<sup>32</sup> bond by human chymase [Nakano *et al* 1997] (Figure 1.1). The discovery of a new isoform of ET may be of clinical importance in conditions where there is systemically increased chymase activity such as CHF [Urata *et al* 1990] and local increases in coronary artery plaque rupture [Kovanen *et al* 1995, Kaartinen *et al* 1994]. *In vitro* studies have shown that ET-1<sub>[1-31]</sub> is vasoactive in several animal vascular beds with a potency similar to ET-1 [Kishi *et al* 1998, Hanson *et al* 1997, Takai *et al* 1998]. In cultured human coronary artery smooth muscle cells ET-1<sub>[1-31]</sub> increases intracellular calcium [Inui *et al* 1999] consistent with the action of vasoactive properties in humans. Its effects in man, *in vivo*, have not been previously reported.

## PROTEASE

## REFERENCE

### Metalloprotease

ECE-1a

Xu *et al* 1994, Ikura *et al* 1994,  
Shimada *et al* 1995

ECE-1b

Schmidt *et al* 1994, Shimada *et al* 1995,  
Valdenaire *et al* 1995

ECE-1c

Schweizer *et al* 1997

ECE-1d

Valdenaire *et al* 1999

ECE-2a

Emoto and Yanagisawa 1995

ECE-2b

Nakahara *et al* 1999

ECE-3

Hasegawa *et al* 1998

SEP

Ikeda *et al* 1999

XCE

Schweizer *et al* 1999

### Aspartyl protease

Pepsin-like

Takaoda *et al* 1990

Cathepsin D,

Sawamura *et al* 1990

pepstatin A-sensitive

Cathepsin D-like, pepstatin

Ikegawa *et al* 1990

A-sensitive

Cathepsin E

Lees *et al* 1990

Pepstatin A-sensitive

Ohnaka *et al* 1990

Pepsin

Pons *et al* 1991

Metal ion aspartic protease,

Wu-wong *et al* 1990, Shiosaki *et al* 1993

pepstatin-like, pepstatin

A-sensitive

Pepstatin A-sensitive,

Knap *et al* 1993

unrelated to cathepsin D

or renin

### Thiol protease

*p*-Hydroxymercuribenzoate sensitive

Deng *et al* 1992

### Serine protease

Chymotrypsin

McMahon *et al* 1989

Chymotrypsin-like

Takaoka *et al* 1990

$\alpha$ -Chymotrypsin

Pons *et al* 1991

Rat lung mast cell chymase I,

Wypij *et al* 1992

chymostatin sensitive

Human mast cell chymase

Nakano *et al* 1997, Kido *et al* 1998

Monkey chymase,

Takai *et al* 1998

chymostatinsensitive

**Table 1.2** List of proteases able to convert ET precursors into active endothelin isoforms (adapted from Endothelin Ligands and their Experimental Effects Within the Human Circulation. Handbook of Physiology. Springer 2001)



### 1.3 ENDOTHELIN RECEPTORS

There are two specific ET receptors (ETRs) in mammals. The ET<sub>A</sub> [Aria *et al* 1990] has a higher affinity for ET-1 and ET-2 whereas the ET<sub>B</sub> [Sakurai *et al* 1990] has equal binding affinity for ET-1, ET-2 and ET-3. The ET<sub>A</sub> and ET<sub>B</sub> receptors, encoded by genes on chromosomes 4 and 13 respectively, are classic G protein coupled receptors with seven hydrophobic helical transmembrane loops [Ogawa *et al* 1991, Elshourbagy *et al* 1992]. There are some functional data suggesting different subtypes of each receptor but this has not yet been confirmed by molecular or genetic studies. In addition, ETRs may undergo post translational modifications such as glycosylation and palmitoylation, which may explain functional differences in disease states. Further differences in physiological and pathophysiological actions may be a result of changes in second messenger signaling systems (see below).

#### 1.3.1 Vascular endothelin receptors

Both the ET<sub>A</sub> and ET<sub>B</sub> receptor types are found on vascular smooth muscle cells, binding to these causes a rise in intracellular calcium and a subsequent contraction. ET<sub>B</sub> receptors are also found on endothelial cells where binding results in a prostanoid and nitric oxide mediated vasodilatation (Figure 1.3).

#### 1.3.2 Cardiac endothelin receptors

Both ET<sub>A</sub> and ET<sub>B</sub> receptors are found in human myocardium [Ponicke *et al* 1998]. However, distribution of the receptor type and number is not uniform and there may be differences in receptor distribution in specific pathological conditions. In situ

hybridization studies have demonstrated both receptor types in atrial and ventricular myocardium, atrioventricular conducting system and the endocardial cell [Molenaar *et al* 1992]. In general, the ratio of ET<sub>A</sub> to ET<sub>B</sub> receptors in terms of number is approximately 60:40% [Peter & Davenport 1996] and receptor density is 1.5 to 2 fold higher in the atrium compared to the ventricle [Ponick *et al* 1998]. In addition, a higher proportion of ET<sub>B</sub> receptors are found in the AV node and the penetrating bundles of His compared with the surrounding myocardium [Molenaar *et al* 1992] suggesting involvement of the ET<sub>B</sub> receptor in neural control of the heart.

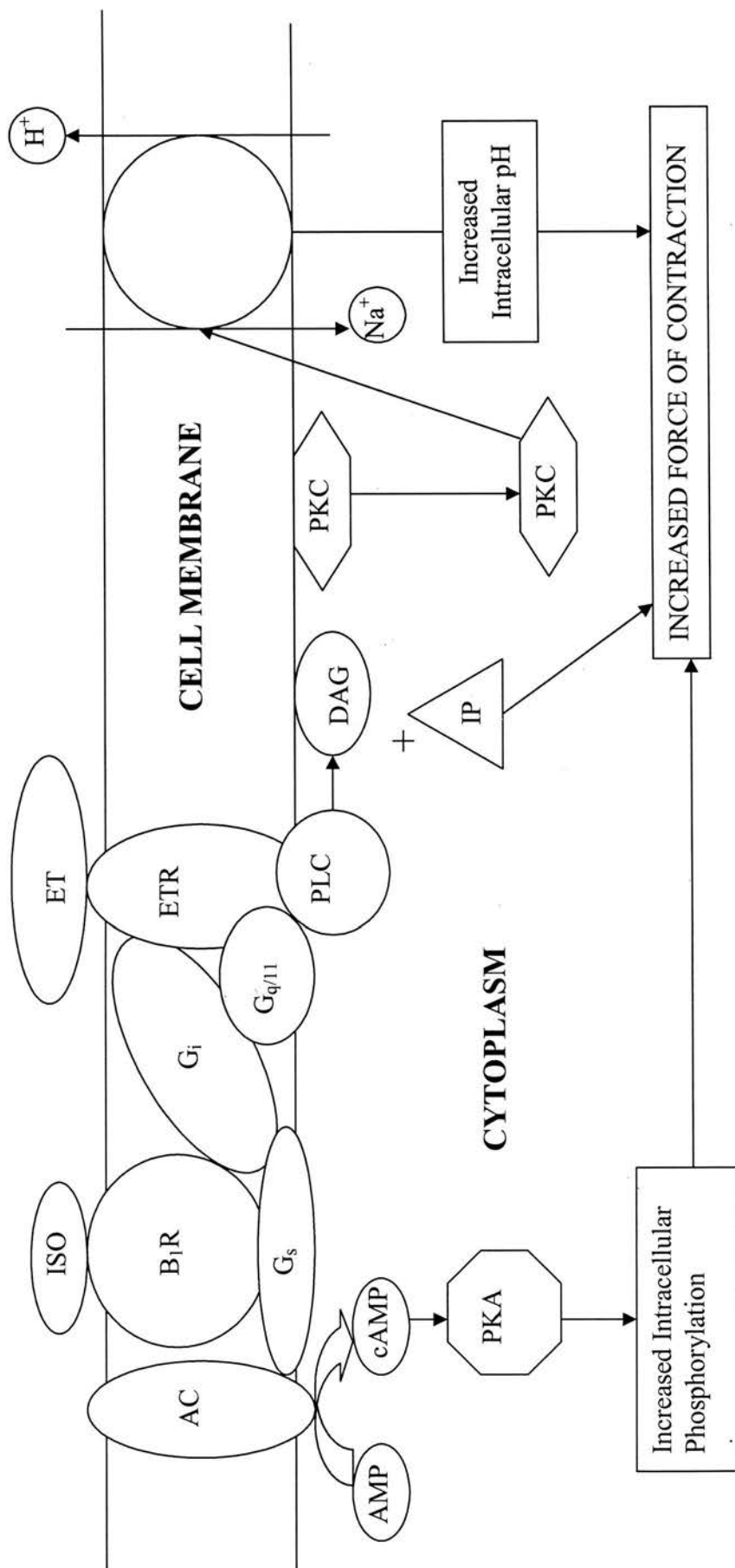
### **1.3.3 Intracellular second messenger signalling**

Binding to the ETR results in activation of a number of intracellular signaling processes. More than one second messenger signalling system may be stimulated simultaneously depending on the cell type and level of expression of G protein subtypes. G protein subtypes coupled to ETRs include G<sub>q</sub>, G<sub>11</sub>, G<sub>s</sub> and G<sub>i2</sub> [Takigawa *et al* 1995]. Downstream signal transduction pathways include enzymes such as nitric oxide synthase, adenylate and guanylate cyclase, protein kinase C, protein tyrosine kinase, and phospholipase A<sub>2</sub>, C and D, ion channels such as calcium and chloride channels and ion transporters (Figure 1.4). Therefore effects of binding to ETRs will also depend on which second messenger pathways are activated and this will change within and between cell types. In addition, many of these second messenger pathways are shared with other neuroendocrine systems and therefore post receptor cross talk may influence responses. One such potential interaction is in the myocardium where beta-adrenergic

stimulation increases adenylate cyclase while endothelin reduces it (Figure 1.4). Thus, there is a potential antagonism between these systems.

#### **1.3.4 Myocardial mast cells**

As previously discussed in this chapter, chymase from human mast cells has been shown to convert big ET-1 to ET<sub>[1-31]</sub> [Kido *et al* 1998]. Mast cells are important mediator of inflammation and are present in human myocardium. Cardiac diseases such as unstable coronary artery disease and heart failure are characterised by local and general increase in inflammatory markers and increased chymase activity. However, the interaction between cardiac mast cells and ET is likely to be complicated. Recent work has demonstrated that ET-1 itself causes mast cell degranulation and down stream release of vasoactive mediators. These mediators could increase coronary arterial tone [Tiefenbacher *et al* 1998] or in the case of human chymase promote local production of ET<sub>[1-31]</sub>.



**Figure 1.4** Diagram of interaction between cardiac ET and beta-adrenergic receptors and second messenger systems. (AC – adenylylase, ISO – isoprenaline, ET – endothelin, ETR – endothelin receptor, PKC – protein kinase C, PKA – protein kinase A, PLC – phospholipase C, IP – inositol triphosphate, DAG – diacyl glycerol, Gi, Gs, Gq/11 – G-proteins.

## 1.4 ENDOTHELIN AGONISTS

Synthetic ET-1, ET-2, ET-3 and sarafotoxin 6c (SX6c), an ET<sub>B</sub> agonist, are available commercially. The ET<sub>A</sub> receptors have a high affinity for ET-1 and ET-2 and lower affinity for ET-3 whereas the ET<sub>B</sub> receptor has equal affinity for ET-1, ET-2 and ET-3. ET-3 and especially SX6c have been used as tools to investigate the ET<sub>B</sub> receptor as ET-3 is 2000 fold selective for the ET<sub>B</sub> receptor and SX6c is 30,000 fold selective for the ET<sub>B</sub> receptor (Table 1.3).

Agonist studies have been useful in defining the target organs and the receptor subtypes involved in physiological responses to the endothelins, however, as the endothelins acts predominantly via autocrine and paracrine mechanisms [Hochoer *et al* 1997], administration of exogenous agonist is unlikely to reproduce physiological responses and results from such studies may be misleading. Therefore, it has been the study of the effects of ETAs which has been central to unravelling the complexities of the pathophysiology of the endothelin system in man (See below).

## 1.5 ENDOTHELIN RECEPTOR ANTAGONISTS

A number of ET receptor antagonists have been developed as research tools and as potential medicines [Douglas *et al* 1997]. Some of these are selective for the ET<sub>A</sub> or ET<sub>B</sub> receptor while others are mixed ET<sub>A/B</sub> receptor antagonists. Some, such as BQ-123, 2000 fold selectivity for the ET<sub>A</sub> receptor [Ihara *et al* 1992] and BQ-788, 200-fold selectivity for the ET<sub>B</sub> receptor [Ishikawa *et al* 1994] are peptides and therefore inactive when given orally (Figure 1.5 a and b). However, other non-peptide ET antagonists such

as bosentan, a mixed  $ET_{A/B}$  receptor antagonist, are orally active, have been developed as potential therapies (Figure 1.5 c). With the current optimism regarding the ET system as a target in a number of pathological conditions, there are many other ET antagonists undergoing pre-clinical and clinical studies [Ferro *et al* 1996, Douglas *et al* 1997]. Details of endothelin antagonists used the studies contained in this thesis are found in Chapter 2.

### 1.5.1 Toxicology of endothelin antagonists

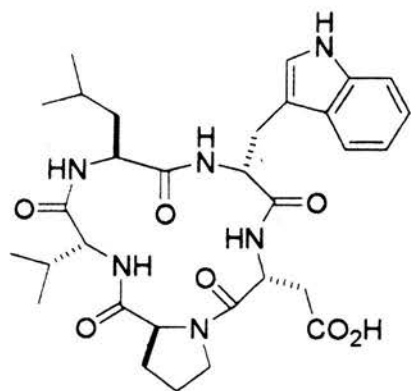
In general endothelin antagonists are well tolerated although they can cause the predicted side-effects of hypotension, nausea and headache [Weber *et al* 1996, Krum *et al* 1998]. Administration of bosentan, a dual ET receptor antagonist, in recent clinical trials, resulted in adverse clinical events such as raised alanine and aspartate aminotransferases, flushing, peripheral oedema and headache [Packer *et al* 1998]. However, there are no data suggesting these effects occur with short term administration of BQ-123 or BQ-788 and the administration of these compounds even in systemic doses in the following studies was extremely well tolerated with no adverse events. The ET system is intimately involved in foetal development and therefore it is not surprising that BQ-123 and BQ-788 have been shown to be teratogenic in animals. In particular, BQ-123 has been shown to increase the incidence of several cardiac outflow tract abnormalities in a mouse model of  $ET_A$  receptor deficiency [Kurihara *et al* 1995]. Due to their, as yet, limited long term usage in clinical trials there are no data on teratogenicity in humans. Therefore only men and post-menopausal women were enrolled in the following studies. In addition, in all of the studies involving intravenous

infusion of BQ-123 or BQ-788, safety blood tests were performed before and after infusion, measuring full blood count, sodium, potassium, urea and creatinine and liver function tests. There were no adverse events in any of the studies.

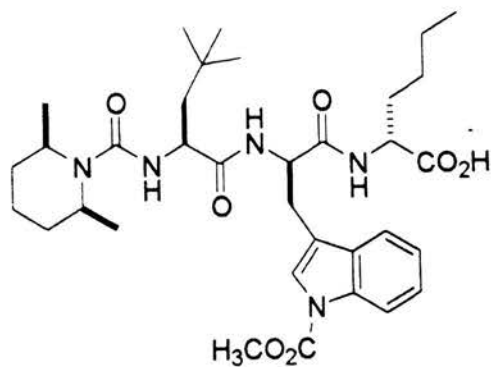
## **1.6 ENDOTHELIN CONVERTING ENZYME INHIBITORS**

There are no selective endothelin converting enzyme (ECE) inhibitors available for clinical studies. In general ECE inhibitors affect many other enzymes to a varying degree, which can confound study results. Two enzyme inhibitors phosphoramidon, a ECE and neutral endopeptidase (NEP) inhibitor and thiorphan, a selective NEP inhibitor have been useful in determining pathways of ET production.

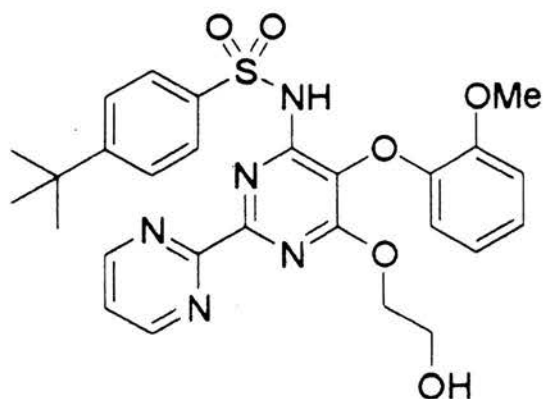
Phosphoramidon (Figure 1.5 d) is a metalloendopeptidase that acts as an ECE inhibitor inhibiting the conversion of big ET to ET-1 [Ikegawa *et al* 1991]. Its principal effect in humans is to cause a decrease in vascular tone, due to inhibition of the endogenous production of ET-1. Local vasodilatation occurs after intra-arterial infusion of locally active doses of phosphoramidon. Intra-arterial infusion of phosphoramidon at concentrations of 30nmol/min has been shown to cause a 40% increase in blood flow in the infused arm with no systemic effects noted [Haynes *et al* 1994b]. Thiorphan (Figure 1.5e) is a selective NEP inhibitor that does not inhibit endothelial cell ECE [Matsumura *et al* 1990, Okada *et al* 1990, Davenport *et al* 1998].



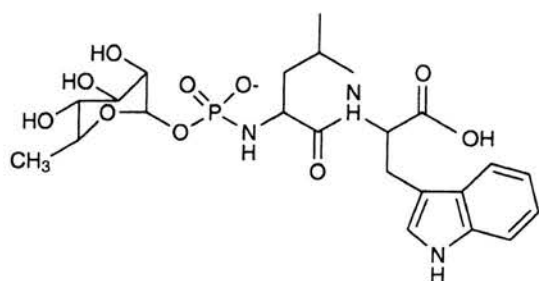
**Figure 1.5 a** – BQ123



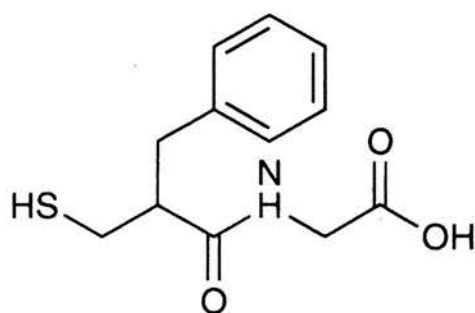
**Figure 1.5 b** - BQ788



**Figure 1.5 c** - Bosentan



**Figure 1.5 d** - Phosphoramidon



**Figure 1.5 e** - Thiorphan

**Figure 1.5** Chemical structures of endothelin antagonists and converting enzyme inhibitors



Potency (EC <sub>50</sub> ,Ki)				
Agonist	Selectivity	ET <sub>A</sub>	ET <sub>B</sub>	Reference
ET-1	ET <sub>A/B</sub>	160 pM	110 pM	Saeki et al 1991
ET-3	ET <sub>B</sub>	140 nM	64 pM	Williams et al 1991
SFTX6c	ET <sub>B</sub>	>7300 nM	0.25 nM	Williams et al 1991
ET-1 <sub>[1-31]</sub>	?ET <sub>A/B</sub>			
Antagonist				
BQ-123	ET <sub>A</sub>	7.3 nM	18 µM	Ihara et al 1991
BQ-788	ET <sub>A</sub>	1.3 µM	1.2 nM	Ishikawa et al 1994
Bosentan	ET <sub>A/B</sub>	4.7 nM	9.5 nM	Clozel et al 1994
TAK044	ET <sub>A/B</sub>	0.1 nM	1.8 nM	Kikuchi et al 1994
Darusantan	ET <sub>A</sub>			

**Table 1.3** Endothelin receptor agonists and antagonists

## **1.7 ENDOTHELIN LIGANDS AND THEIR EXPERIMENTAL EFFECTS IN HEALTHY VOLUNTEERS**

### **1.7.1 Local ET activation in healthy volunteers**

#### **1.7.1.1 Resistance vessels**

Infusion of ET-1 (5 pmol/min) into the brachial artery caused a slow onset, dose-dependent sustained reduction in blood flow of 40% at 60 min in the forearm of healthy volunteers. This persisted for up to 2 hours following discontinuation of the infusion [Clark *et al* 1989]. Similar vasoconstriction has been demonstrated by other groups and in addition ET-1 caused a non-significant trend towards causing early vasodilatation [Haynes *et al* 1995a]. In one study, sustained vasodilatation has been described with ET-1 at low concentrations (0.2 pmol/min/100 ml forearm tissue) [Kiowski *et al* 1990]. The low doses used in this study may have been insufficient to cause the sustained vasoconstriction response seen in other studies but at the same time sufficient to cause the initial vasodilatation. The mechanisms behind this are not clearly understood.

SX6c and ET-3, both ET<sub>B</sub> agonists, also caused constriction of forearm resistance vessels *in vivo* but to a lesser degree than ET-1 [Haynes *et al* 1995b], thus implicating the ET<sub>B</sub> receptor in vasoconstriction. However, ET-1, ET-3 and SX6c caused a transient vasodilatation prior to a sustained vasoconstriction. The transient vasodilatation is more marked with ET-3 and SX6c suggesting that this is mediated via endothelial ET<sub>B</sub> receptors [Haynes *et al* 1995b]. Thus, it seems that ET<sub>B</sub> receptors can mediate both vasodilatation and vasoconstriction.

It is postulated that the ET<sub>B</sub> receptors on the vascular smooth muscle cells cause vasoconstriction [Williams *et al* 1991, Moreland *et al* 1992, Sumner *et al* 1992] while the endothelial cell receptors mediate vasodilatation [Takayanagi *et al* 1991] possibly also acting as clearance receptors for ET-1.

The exact mechanism of ET-3 and SX6c vasoconstriction is unclear. It may be mediated by direct binding to the ET<sub>B</sub> receptors on the vascular smooth muscle cells resulting in vasoconstriction. However, it may be that ET<sub>B</sub> binding causes displacement of ET-1 thus increasing stimulation at the ET<sub>A</sub> receptor resulting in vasoconstriction. In addition, there is animal evidence that SX6c may act through non-endothelin dependent mechanisms, and the pressor responses may be independent of the endothelin system [Flynn *et al* 1995].

Local infusions of big ET-1 (50 pmol/min) produce vasoconstriction which can be blocked by phosphoramidon, a combined ECE and NEP inhibitor [Haynes *et al* 1994b]. There is limited plasma ECE activity [Watanabe *et al* 1991] and this result, therefore, suggests that forearm resistance vessels have local ECE activity.

#### **1.7.1.2 Capacitance vessels**

Constriction of dorsal hand veins is seen with ET-1 infusions of 5 pmol/min [Clark *et al* 1989, Haynes *et al* 1993a, 1995b]. There is no venoconstriction to local infusion of big ET-1 (50 pmol/min) suggesting no local ECE activity in hand veins [Haynes *et al* 1995b]. The mechanism of action of ET-1 in hand veins is thought to be mediated via both

the opening of voltage operated  $\text{Ca}^{2+}$  channels and the closure of ATP sensitive  $\text{K}^{+}$  channels thus offering other targets for therapeutic intervention [Haynes *et al* 1993b]. Sarafotoxin (SX6c) and ET-3 ( $\text{ET}_B$  agonists) also caused constriction of hand capacitance vessels *in vivo* but to a lesser degree than ET-1 [Haynes *et al* 1995b], thus implicating the  $\text{ET}_B$  receptor in vasoconstriction.

Dorsal hand veins have no intrinsic tone, however, in precontracted human hand veins, as in arteries, ET-1, ET-3 and SX6c also caused a transient vasodilatation prior to a sustained vasoconstriction. The transient vasodilatation was more marked with ET-3 and SX6c suggesting that it is mediated via endothelial  $\text{ET}_B$  receptors [Haynes *et al* 1995b]. This effect is blocked by Aspirin and therefore it is postulated that it is prostanoid dependent [Haynes *et al* 1994a].

#### **1.7.1.3 Microcirculation**

Intra-dermal injection of ET-1 results in vasoconstriction of the microcirculation [Wenzel *et al* 1994]. Intra-dermal ET-3 injection does not cause vasoconstriction suggesting that vasoconstriction in the skin microcirculation is an  $\text{ET}_A$  mediated response. More recently, studies have demonstrated vasodilatation, 1-2 cm from the site of injection [Wenzel *et al* 1998a]. It appears that this is an  $\text{ET}_A$  receptor mediated effect through stimulation of polymodal nociceptor fibres leading to nitric oxide release because this effect is blocked by BQ-123 and pre-treatment with L-NMMA, lignocaine and capsaicin. This potentially, implicates endothelins in the process of neurogenic

inflammation, suggesting ETAs may possibly have a role to play in the treatment of inflammatory conditions.

The overall effect of ET-1 at the ET<sub>B</sub> receptor will therefore be determined by the balance of effects between its actions at the endothelial and vascular smooth muscle ET<sub>B</sub> receptors. As previously discussed, the results of agonist studies should be interpreted with caution as the endothelins act in a paracrine and autocrine fashion and the administration of supra-physiological concentrations of exogenous agonists may not mimic *in vivo* physiology. The results of antagonist studies are likely to be much more illuminating.

## **1.7.2 Local ET inhibition in health volunteers**

### **1.7.2.1 Resistance vessels**

Local infusion of BQ-123 [Haynes *et al* 1994a, Berrazueta *et al* 1997, Verhaar *et al* 1998] and TAK-044 [Haynes *et al* 1996] results in vasodilatation in the forearm arteries of healthy volunteers. The larger effect seen with BQ-123 suggests that vasoconstriction to ET-1 is mediated predominantly through the ET<sub>A</sub> receptor located on vascular smooth muscle cells. Thus, the net effect of inhibition of the ET<sub>B</sub> receptor is vasoconstriction. The role of the ET<sub>B</sub> receptor is further clarified by studies with the ET<sub>B</sub> receptor selective antagonist, BQ-788 [Ishikawa *et al* 1994]. BQ-788 causes vasoconstriction in the forearm vessels of healthy volunteers [Verhaar *et al* 1998]. This vasoconstriction persists on a background of ET<sub>A</sub> antagonism [Verhaar *et al* 1998], reinforcing the hypothesis that ET-1 stimulation of the endothelial ET<sub>B</sub> receptor causes dilatation, and is

likely to be a direct effect, whereas the vascular smooth muscle ET<sub>B</sub> receptor causes vasoconstriction.

#### **1.7.2.2 Microcirculation**

In the skin microcirculation, in healthy volunteers, intradermal injection of a mixed ET<sub>A/B</sub> antagonist caused a vasodilatation which was no greater than that seen with a selective ET<sub>A</sub> antagonist suggesting vasoconstriction to endothelin is solely ET<sub>A</sub> receptor mediated [Wenzel *et al* 1994]. However, in patients with coronary artery disease there was increased vasodilatation with mixed ET<sub>A/B</sub> antagonism over ET<sub>A</sub> antagonism, suggesting there may be ET<sub>B</sub> receptor mediated vasoconstriction in these patients [Wenzel *et al* 1996]. In addition, intravenous administration of Bosentan reversed the vasoconstrictor effect of ET-1 measured in the skin microcirculation [Weber *et al* 1996]. This study did not report a distal flare following intra-dermal ET-1 administration. However, in a more recent study, pretreatment with intra-dermal ET<sub>A</sub> receptor antagonist prevented the ET-1 induced vasoconstriction and also the 'flare reaction' caused by vasodilatation in the surrounding area. There was no additional effect with mixed ET<sub>A/B</sub> antagonist suggesting that the flare reaction is an ET<sub>A</sub> receptor mediated response [Wenzel *et al* 1998]. Thus, in the skin microcirculation in healthy volunteers, ET<sub>A</sub> receptors appear to be involved in ET mediated vasoconstriction and to mediate the distal vasodilatation.

#### **1.7.3 Systemic ET activation in healthy volunteers**

#### **1.7.3.1 Cardiovascular effects**

The administration of ET-1 (5ng/kg/min for 15 min) to healthy volunteers results in an increase in mean blood pressure of 5-10 mmHg and a reduction in heart rate, which is probably reflex in nature. This dose of ET-1 increased plasma concentrations by 50 fold [Vierhapper *et al* 1990]. In more recent studies, both ET-1 (8 pmol/kg/min for 10 min) and big ET (8 pmol/kg/min) infusions, sufficient to cause increases in plasma ET-1 of 30 and 2.4 fold respectively, caused similar increases in blood pressure (BP) and a reduction in heart rate which persisted following cessation of the infusion for 30 and 90 min respectively. These doses of ET-1 and big ET-1 also reduced coronary sinus blood flow, by a maximum of ~25%, and increased coronary vascular resistance by ~50 and 100% respectively [Pernow *et al* 1996]. In other studies, similar haemodynamic changes following big ET-1 infusion (8 pmol/min for 20 min; 2 fold increase in plasma ET-1) persisted for up to 2 hours [Ahlborg *et al* 1994]. Further studies have shown that doses of ET-1 insufficient to cause systemic or pulmonary pressor effects (0.75 pmol/kg/min) can impair left and right diastolic dysfunction and are negatively inotropic [Kiely *et al* 1997].

#### **1.7.3.2 Renovascular effects**

A reduction in splanchnic and renal blood flow by 34 and 26% respectively is seen following ET-1 infusion (4 pmol/kg/min for 20 min; 12 fold increase in plasma ET-1) which persisted for 1 and 3 hours respectively [Weitzberg *et al* 1991]. More recently, changes in renal parameters have been demonstrated following administration of lower doses of ET-1 (1 pmol/kg/min for 60 min: 11 fold increase in plasma ET-1) to healthy

volunteers causing an increase in diastolic BP of 8% and decrease in heart rate of 14% but no significant change in systolic BP. This reduction in heart rate is probably 'reflex' in response to reduced systemic vascular resistance. Renal plasma flow and glomerular filtration rate (GFR) were both reduced, by ~35 and 16% respectively. Urine flow was reduced by 40% and urinary sodium excretion by 58%. Lithium clearance in these subjects suggested that the reduction in sodium excretion occurred at the distal rather than proximal tubule [Sorensen *et al* 1994]. Infusion of lower doses of ET-1 (0.4 pmol/kg/min) for a longer period (6 hours) results in decreased renal blood flow by 43%, associated with a 10% decrease in heart rate. This dose of ET-1 increased plasma ET concentration by ~300% [Jilma *et al* 1997]. In a similar study the administration of exogenous ET-1 (2.5ng/kg/min) to healthy volunteers sufficient to cause a 3 fold increase in plasma ET concentrations caused renal vasoconstriction and sodium retention. In this study, administration of a comparable dose of ET-3 produced no effect on blood pressure, renal blood flow or electrolyte excretion suggesting that these responses are predominantly ET<sub>A</sub> mediated [Kaasjager *et al* 1997]. Interestingly, ET-1 produced sodium retention in humans even at doses insufficient to reduce renal plasma flow or glomerular filtration rate [Rabelink *et al* 1994] suggesting that ET may have subtle intra-renal effects on sodium handling.



## **1.7.4 Systemic ET inhibition in healthy volunteers**

### **1.7.4.1 Cardiovascular effects**

The results of systemic studies with ETAs have, in general, confirmed the predictions made from the results of local vascular studies.

#### **1.7.4.1.1 Non-selective inhibition**

The administration of the ET<sub>A/B</sub> antagonist TAK-044 (1000 mg over 15 min) reduced systolic blood pressure, diastolic blood pressure and systemic vascular resistance by 4%, 18% and 26% respectively and resulted in an increase in both heart rate and cardiac index. Plasma ET concentrations increased by 9-fold following administration of TAK-044 (1000mg). These results suggest an effect predominantly in resistance vessels [Haynes *et al* 1996]. The increase in plasma ET is thought to be the result of displacement of bound ET-1 and reduced ET-1 clearance as there is no associated increase in big ET-1 or its C-terminal fragment [Plumpton *et al* 1996]. Similar results are found in healthy volunteers given oral and intra-venous bosentan. Bosentan, 2400 mg administered orally, resulted in a maximal decrease in systolic blood pressure of 9 mmHg two hours after dosing [Weber *et al* 1996]. This study also demonstrated a dose-dependent increase in plasma ET-1 after administration of bosentan; 2-fold following 2400 mg (oral) and 3-fold following 750 mg (IV).

#### **1.7.4.1.2 Selective inhibition**

BQ-123 (3000 nmol/min for 15 min) administered to healthy volunteers resulted in a reduction in mean arterial blood pressure and systemic vascular resistance index of 12%

and 23% respectively with an associated increase in cardiac index and a non significant increase in heart rate. Similar effects were seen with lower doses (1000 nmol/min for 15 min) [Spratt *et al* 2001]. Forearm vasoconstriction to local infusions of ET-1 is blocked with the higher systemic doses of BQ-123 (3000nmol/min for 15 min), in this study, thus confirming the dominant role of the ET<sub>A</sub> receptor as a vasoconstrictor in peripheral resistance vessels. Interestingly, there was no consistent increase in plasma big ET-1 or plasma ET-1 with systemic blockade of the ET<sub>A</sub> receptor.

The administration of BQ-788, a selective ET<sub>B</sub> receptor blocker, at systemic doses (300 nmol/min for 15 min) to healthy men causes systemic vasoconstriction with a reduction of heart rate and cardiac index but no effect on mean arterial blood pressure, again suggesting that endogenous activity at the vascular ET<sub>B</sub> receptors mediates a predominantly vasodilator tone [Strachan *et al* 1999]. In common with previous studies plasma ET-1 increased, 2-fold, with ET<sub>B</sub> receptor blockade.

#### **1.7.4.2 Renovascular effects**

BQ-123 (~100 nmol/min IV for 60 min) administered to healthy men had no renal or systemic effects but did prevent ET-1 (1 pmol/kg/min for 120 min) induced reductions in renal plasma flow and GFR [Schmetterer *et al* 1998]. These results suggest that the vasoconstriction of renal vessels caused by ET-1 are mediated through the ET<sub>A</sub> receptor and are more sensitive to ET<sub>A</sub>Rs than the peripheral vasculature. The authors claim that the lack of change in renal parameters following administration of BQ-123 suggests there is no ET mediated resting tone in the renal vasculature. However, despite reversing

the effects of exogenous ET-1, the lack of a systemic effect with this dose of BQ-123 would suggest that this dose may be insufficient to cause renal effects.

More recently, administration of the mixed ET<sub>A/B</sub> receptor antagonist, TAK-044 resulted in a decrease in effective renal vascular resistance, suggesting there may be ET mediated resting vascular tone in the kidney. However there was a decrease in GFR and a significant decrease in the filtration fraction with both doses of TAK-044 [Ferro *et al* 1998].

Thus, the question as to whether there is basal endothelin mediated tone and the role of the ET<sub>A</sub> and ET<sub>B</sub> receptor in the renal vasculature remain to be clarified.

## **1.8 ENDOTHELIN LIGANDS AND THEIR EFFECTS IN PATIENTS**

### **1.8.1 Heart disease**

#### **1.8.1.1 *Chronic heart failure***

The first clinical trial of an ET antagonist in CHF involved the systemic administration of bosentan (100 mg IV followed by 200 mg IV after 60 min) and resulted in a reduction of mean arterial pressure, systemic and pulmonary vascular resistance and an increase in cardiac output without reflex increases in heart rate and importantly no increase in plasma angiotensin II or noradrenaline [Kiowski *et al* 1995]. Pulmonary vascular resistance was decreased to a greater degree than systemic vascular resistance, in contrast to the effect usually seen with other vasodilators. These patients, however, do

not reflect the general patient population as they were not being treated with angiotensin converting enzyme (ACE) inhibitors.

Subsequently, local studies were performed in CHF patients on standard treatments (including ACE inhibitors). Local brachial artery infusion of ET-1 (5 pmol/min for 60 min) caused less vasoconstriction compared with matched controls in resistance [Love *et al* 1996b] and capacitance vessels [Love *et al* 1996c]. In these patients potentially beneficial haemodynamic effects are seen with local brachial artery infusion of BQ-123 (100 nmol/min for 60 min) which causes vasodilatation. Although there is increased vasoconstriction to the ET<sub>B</sub> receptor agonist, S6Xc (5 pmol/min for 60 min), in CHF patients when compared with matched controls, BQ-788 also causes vasoconstriction, suggesting the net effect of stimulation of the ET<sub>B</sub> receptor is vasodilatation. The effects of S6Xc have been discussed previously.

Systemic studies in patients on treatment including ACE inhibitors, digitalis glycosides and diuretics demonstrating similar haemodynamic improvements in the short term with a small increase in heart rate when given bosentan orally (0.5g twice a day for 14 days) [Sutsch *et al* 1997]. A further study demonstrated improved haemodynamic parameters in CHF patients treated with oral bosentan (1g twice daily) with an increase in cardiac output of 15% and a decrease in systemic and pulmonary vascular resistance each of ~10% respectively. This is important, as these beneficial effects were seen in patients already treated with ACE inhibitors, diuretics and digoxin [Sutsch *et al* 1998].

More recently the results from a larger study, REACH-1, were reported. 370 patients with NYHA class IIIB-IV on standard treatments, including ACE inhibitors, received either bosentan 500 mg bd or placebo for 6 months. The bosentan group had a reduction in the numbers of hospital admissions for any reason by 41%. The trial was stopped early due to concerns over raised hepatic transaminases. However, in patients followed for the intended duration of the study (6 months) bosentan significantly increased the likelihood of clinical improvement and decreased the likelihood of CHF deterioration [Packer *et al* 1998].

These studies have demonstrated the beneficial haemodynamics of short term non-selective ET receptor blockade in CHF patients. In order to investigate whether selective antagonism will confer clinical benefit, BQ-123 (200 nmol/min for 60 min) was administered by intravenous infusion to 10 CHF patients. This caused a systemic vasodilatation and rise in cardiac index (CI), with no change in heart rate (HR). There was a non-significant fall in pulmonary vascular resistance (PVR) [Cowburn *et al* 1998a].

In further studies, consistent with predictions from local studies in CHF patients [Love *et al* 1996a and 1996b], effects of a selective ET<sub>B</sub> receptor antagonist in 8 CHF patients were investigated. BQ-788 (50-100 nmol/min for 45 min) caused systemic vasoconstriction, a rise in MAP and SVR, with a reduction in cardiac index. The subsequent infusion of the selective ET<sub>A</sub> receptor antagonist BQ-123 resulted in a reversal of these effects and an increase in cardiac index [Cowburn *et al* 1998b].

A further study of 24 CHF patients with the selective ET<sub>A</sub> antagonist TBC11251 (3mg/kg/min for 15 min) demonstrate a significant fall in mean pulmonary artery pressure of 12% and PVR of 39% following systemic administration of the study drug for 15 min. An effect was seen at 15 min, becoming significant at 1 hour and maximal at 2-3 hours. Conversely, there was no significant change in HR, MAP, SV, pulmonary capillary wedge pressure (PCWP) or SVR [Givertz *et al* 1998].

Thus, the evidence currently suggests that selective ET<sub>B</sub> receptor blockade has deleterious effects on the haemodynamics of patients with CHF. However, it is still unclear, when the ET<sub>A</sub> receptor is blocked, whether ET<sub>B</sub> blockade will improve systemic haemodynamics in these patients.

#### **1.8.1.2 Coronary artery disease**

There have been few studies performed specifically in patients with coronary artery disease (CAD). In healthy volunteers, intradermal injection of mixed ET<sub>A/B</sub> receptor antagonist caused a similar vasodilatation compared with selective ET<sub>A</sub> receptor antagonism in the skin microcirculation. However, in patients with CAD, mixed inhibition caused a greater vasodilatation [Wenzel *et al* 1996]. This suggests that the ET<sub>B</sub> receptor may have increased functional significance in patients with CAD.

In a recent systemic study, 28 patients with angiographically documented coronary artery disease were given bosantan 200 mg IV. As may have been predicted from

previous studies, this resulted in a decrease in systolic blood pressure and a small increase in heart rate. In addition, there was an increase in coronary artery diameter which appeared to be maximal as no further increase was noted after treatment with glyceryl trinitrate. This suggests there is a basal coronary artery vasoconstrictor tone, *in vivo*, mediated by endogenous ET [Wenzel *et al* 1998b]. This has been confirmed by another study demonstrating endogenous ET<sub>A</sub> receptor mediated coronary artery tone in patients undergoing coronary arteriography [Kyriakides *et al* 2002].

#### **1.8.1.3 Hypertension**

In patients with essential hypertension there is an increased venoconstrictor response to local ET-1 (5 pmol/min) and sympathetically mediated venoconstriction of capacitance vessels is potentiated by ET-1 [Haynes *et al* 1994a]. Vasodilatation in patients with essential hypertension following intra-arterial administration of BQ-123 was shown to be no different to that in normal healthy volunteers, suggesting that there may be no major dysfunction of endothelium dependent vasodilatation [Ferro *et al* 1996b]. Several animal and *in vitro* experiments on human tissue have demonstrated close interactions between the endothelin, renin-angiotensin and sympathetic nervous systems in several disease states although there is limited human *in vivo* data.

Results of systemic studies performed with hypertensive patients have recently been reported. Bosentan treatment for 4 weeks in 293 hypertensive patients caused significant lowering of blood pressure without reflex neurohormonal stimulation of the sympathetic nervous system or the renin-angiotensin system. Observation of the effects of 4 doses of

bosentan demonstrated a plateau reached by 500 mg of bosentan which was similar to the reduction in blood pressure seen with 20 mg of the ACE inhibitor enalapril [Krum *et al* 1998].

## **1.8.2 Renal disease**

### **1.8.2.1 Chronic renal failure**

Compared with the vast animal literature concerning endothelin ligands and their effects on the renal system, there are relatively few human data. Plasma concentrations of ET-1 are raised in chronic haemodialysis patients and in a recent study of precontracted hand veins, in this group, infusion of BQ-123 (3mg/kg/min for 45 min) caused an increase in venodilation of 74% compared with 28% in controls suggesting that responses to endogenous ET may be increased in patients with CRF [Bussemaker *et al* 1998]. Administration of the mixed ET<sub>A/B</sub> receptor antagonist, TAK-044 (100mg and 750mg IV over 15 min) resulted in a reduction of MAP and SVRI of 11% and 24% respectively and an increase in cardiac output. TAK-044 at both doses had no significant effect on GFR or effective renal plasma flow. However, effective renal vascular resistance was lowered by ~10% by both doses. Neither dose had any effect on sodium or lithium clearance [Ferro *et al* 1998]. This study suggests that there is ET mediated renovascular tone and that ETAs reduce blood pressure in CRF and may have a potential beneficial role as vasodilators in these patients.



### **1.8.2.2 Hepatorenal syndrome**

The hepatorenal syndrome (HRS) is characterised by renal vasospasm in the face of systemic vasodilatation resulting in sodium retention which persists despite adequate correction of plasma volume and cardiac output. Plasma ET-1 concentrations are increase in this condition even when compared with patients with hepatic and renal failure of different aetiology. Recently, in a small study, 3 patients with HRS received BQ-123 (10, 25 and 100 nmol/min for 60 min). There was a dose dependent increase in renal plasma flow and glomerular filtration rate as measured by PAH and inulin clearance but no significant changes in HR, MAP or SVR [Soper *et al* 1996]. These results correspond well with the results of studies in healthy volunteers [Rabelink *et al* 1994].

### **1.8.2.3 Contrast nephropathy**

Despite previous suggestions that contrast-stimulated, endothelin mediated intrarenal vasoconstriction may contribute to contrast nephrotoxicity, a recent study of 158 high risk patients undergoing coronary angiography demonstrated that the non-selective endothelin antagonist SB 209670 did not protect against contrast nephropathy. Indeed, more patients in the treatment group compared with the control group developed contrast nephropathy (56% vs 29%) [Wang *et al* 1998].

## 1.9 CONCLUSIONS

The endothelin system has been extensively studied over the last 10 years. Much of our understanding of the role of endothelin in normal physiology and pathophysiology comes as a result of carefully designed clinical studies with ETAs. In addition to their use in endothelin research, ETAs are now being developed as potential therapeutic agents in various cardiovascular conditions. It appears that they may be of most use in clinical conditions which result in chronic vasoconstriction, such as heart failure and systemic and pulmonary hypertension, as well as in conditions resulting from vasospasm such as subarachnoid haemorrhage. However, the best target for these potential medicines is not yet known, and it is unclear whether mixed ET<sub>A/B</sub> antagonism or selective ET<sub>A</sub> antagonism will provide the best clinical treatment, and indeed, this may differ between conditions. In addition, it remains unclear whether it will be possible to selectively target the endothelial or vascular smooth muscle ET<sub>B</sub> receptor.

In conclusion, there is a need for continuing research to further define the roles of the ET<sub>A</sub> and ET<sub>B</sub> receptors in health and disease and for well designed clinical trials to confirm whether ETAs provide benefits in terms of morbidity and mortality in different target populations.

## 1.10 HYPOTHESES AND AIMS

Endothelin antagonists are being developed as potential therapies in several conditions, however it is still not clear whether selective ET<sub>A</sub> antagonism will be better than dual ET<sub>A/B</sub> antagonism, and indeed this may differ between conditions. Endothelin-1<sub>[1-31]</sub> is a newly discovered isoform of endothelin produced by the action of human mast cell chymase on big ET-1. This may represent a novel pathway of ET-1 production although animal studies suggest that ET-1<sub>[1-31]</sub> is vasoactive. This thesis will address the following hypotheses.

**Hypothesis 1: The skin microcirculation is a useful tool for investigating the effects of vasoactive substances, *in vivo*, in humans.**

The investigation of new, potentially harmful, vasoactive substances, *in vivo*, in humans requires safe techniques. The skin microcirculation offers an isolated vascular bed where substances can be safely investigated using small doses of study compound. The safety, tolerability and repeatability of this technique were assessed (Chapter 3). In addition, the effects of endothelin agonist and antagonists with known vascular effects in other vascular beds were investigated. (Chapter 6)

**Hypothesis 2: The Filtrass 2001 is a more accurate and repeatable device for measuring forearm blood flow than the established Hokanson EC4.**

Forearm plethysmography is a useful tool for investigating the vasoactive properties of compounds in resistance blood vessels. Recently, a new device has been developed which offers potential advantages over existing devices. However, whether this device

offered significant advantages over the established Hokanson EC4 for future studies was unknown. This device was tested against the existing and well validated Hokanson EC4 (Chapter 4).

**Hypothesis 3: Thoracic Bioimpedance can be used in controlled studies in CHF patients.**

Due to its non-invasive nature, Thoracic Bioimpedance (TB) has found utility in clinical studies on healthy volunteers. However, its use in clinical practice, especially in unstable patients, has been questioned. Its utility in patients with stable heart failure within a controlled haemodynamic study is not known. TB was compared against the more invasive thermodilution technique in patients undergoing a systemic clinical study (Chapter 10) to determine if it could be used in future studies (Chapter 5).

**Hypothesis 4: Endothelin-1<sub>[1-31]</sub> is vasoactive in the human skin microcirculation.**

The above hypothesis was tested in the skin microcirculation of healthy volunteers (Chapter 6).

**Hypothesis 5: Plasma concentrations of endothelin-1<sub>[1-31]</sub> are elevated in patients with chronic heart failure.**

Endothelin-1<sub>[1-31]</sub> is formed by the action of human chymase on big ET-1. Human chymase activity is increased in chronic heart failure. A new commercially available EIA kit for endothelin-1<sub>[1-31]</sub> was evaluated and used to investigate the above hypothesis in the plasma of patients with chronic heart failure (Chapter 7).

**Hypothesis 6: Endothelin-1 is positively inotropic and antagonises beta-adrenergic stimulation of isolated human myocardium.**

Both ET-1 and beta adrenoceptor antagonists have previously be shown to be positively inotropic in isolated human. However, in myocardial membrane preparations beta-adrenergic stimulation increases adenylate cyclase activity while endothelin reduces it. The functional consequences of this potential antagonism were investigated (Chapter 8).

**Hypothesis 7: Endothelin-1 is positively inotropic and contributes to resting force of contraction in isolated human myocardium.**

Endothelin-1 has previously been shown to cause increased force of contraction in isolated human myocardium. The effects of endothelin agonism and antagonism on resting myocardial force of contraction were investigated (Chapter 8).

**Hypothesis 8: Treatment of hypercholesterolaemia will reduce arterial stiffness and increase ET mediated vasodilatation.**

Hypercholesterolaemia is a risk factor for atherosclerosis. It is associated with impairment of nitric oxide mediated vasodilatation and which may be improved by treatment with lipid lowering therapy. The effects of statin therapy on the endothelin responses of resistance blood vessels in healthy volunteers with hypercholesterolaemia has not previously been addressed (Chapter 9).

**Hypothesis 9: Selective ET<sub>A</sub> antagonism will result in different haemodynamic changes than dual ET<sub>A/B</sub> antagonism in patients with chronic heart failure.**

Chronic heart failure is associated with an increase in plasma ET and changes in ET receptor number and function. Previous studies have demonstrated haemodynamic improvements with both selective and dual ET<sub>A/B</sub> antagonist. Furthermore, selective systemic ET<sub>B</sub> antagonism has been shown to result in adverse haemodynamic changes, a direct comparison between selective ET<sub>A</sub> antagonism and dual ET<sub>A/B</sub> antagonism in patients with chronic heart failure has not previously reported (Chapter 10).

## **Chapter 2**

### **Methodology**

**Leslie SJ, Webb DJ.** Techniques used in the Clinical Investigation of the Cardiovascular System. *The Bulletin of the British Society for Cardiovascular Research* 2000;13:4-8.

## 2.1 INTRODUCTION

Several *in vitro* and *in vivo* techniques for the investigation of the cardiovascular system in humans have been employed in the generation of this thesis. The general principles and details have been outlined below with details specific to each study outlined in the methods sections of subsequent chapters.

### 2.1.1 *In vivo* vs *in vitro* techniques

In general, with some obvious limitations, *in-vivo* techniques offer an advantage over *in-vitro* techniques when investigating cardiovascular responses to vasoactive compounds in humans as vessels are operating under physiological conditions. However, controlled manipulations of the surrounding milieu may, in some cases, make *in-vitro* techniques preferable. In this thesis, isolated human myocardial trabeculae were used to study the direct functional effects of ET and ETRAs on the human myocardium isolated from confounding physiological compensatory mechanism.

### 2.1.2 Systemic vs local *in vivo* techniques

Systemic haemodynamic changes, in response to systemic drug dosing, can be measured by invasive methods such as by pulmonary artery catheterisation or non-invasive methods such as pulse wave analysis and thoracic bioimpedence. However, some vascular beds are more sensitive than others to the effects of vasoconstrictor substances (e.g. renal, coronary and cerebral circulations) and therefore there are potential risks when administering vasoactive substances at systemic doses. Local study techniques such as forearm plethysmography coupled with brachial artery infusion or laser Doppler



skin flowmetry coupled with intradermal injection, can be used to investigate responses to compounds in single vascular beds. These techniques have the advantage that they allow the *in-vivo* investigation of compounds while using doses which are 10-1000 times lower than those required to give a systemic effect [Benjamin *et al* 1995, Webb 1995]. In addition, to increased safety, the use of small and locally active doses minimises potentially confounding compensatory effects of the heart, kidney and nervous system which can occur with systemic dosing. However, ultimately the systemic effect of compounds require to be investigated prior to large scale clinical trials to determine their clinical utility.

### **2.1.3 Ethical considerations**

All studies were performed with the approval of the Lothian Research Ethics Committee in accordance with the Declaration of Helsinki of the World Medical Association. Volunteers and patients, were given as long as required, and at least 24 hours, to decide whether they wished to take part in the study. Written informed consent was obtained from each volunteer or patient before entry to the study.

### **2.1.4 Subject recruitment**

Recruitment procedures differed between studies. For healthy volunteer studies the healthy volunteer database at the Clinical Research Centre, Western General Hospital was used. Hypercholestreolaemic subjects were identified through a systematic screening programme of staff and volunteers at the Western General Hospital. Myocardial tissue was taken from patients undergoing a cardiopulmonary bypass

procedure, identified from theatre lists and approached on Ward 17 of the Edinburgh's Royal Infirmary. Patients with left ventricular dysfunction were identified from the echocardiography database followed by case note review at Edinburgh's Western General Hospital. Patients were then contacted by letter to ask them if they wished to participate.

#### **2.1.5 Subject preparation**

None of the healthy volunteers were taking regular medication and avoided any medication for 1 week prior to study. All subjects and patients abstained from alcohol for 24 hours and from food, tobacco and caffeine containing drinks for at least 5 hours before each study. Studies were performed in a quiet temperature controlled, draft free, room (23-25 °C). All studies were preceded by at least 30 min of baseline measurements (90 min in invasive haemodynamic studies).

### **2.2 SYSTEMIC HAEMODYNAMIC STUDIES**

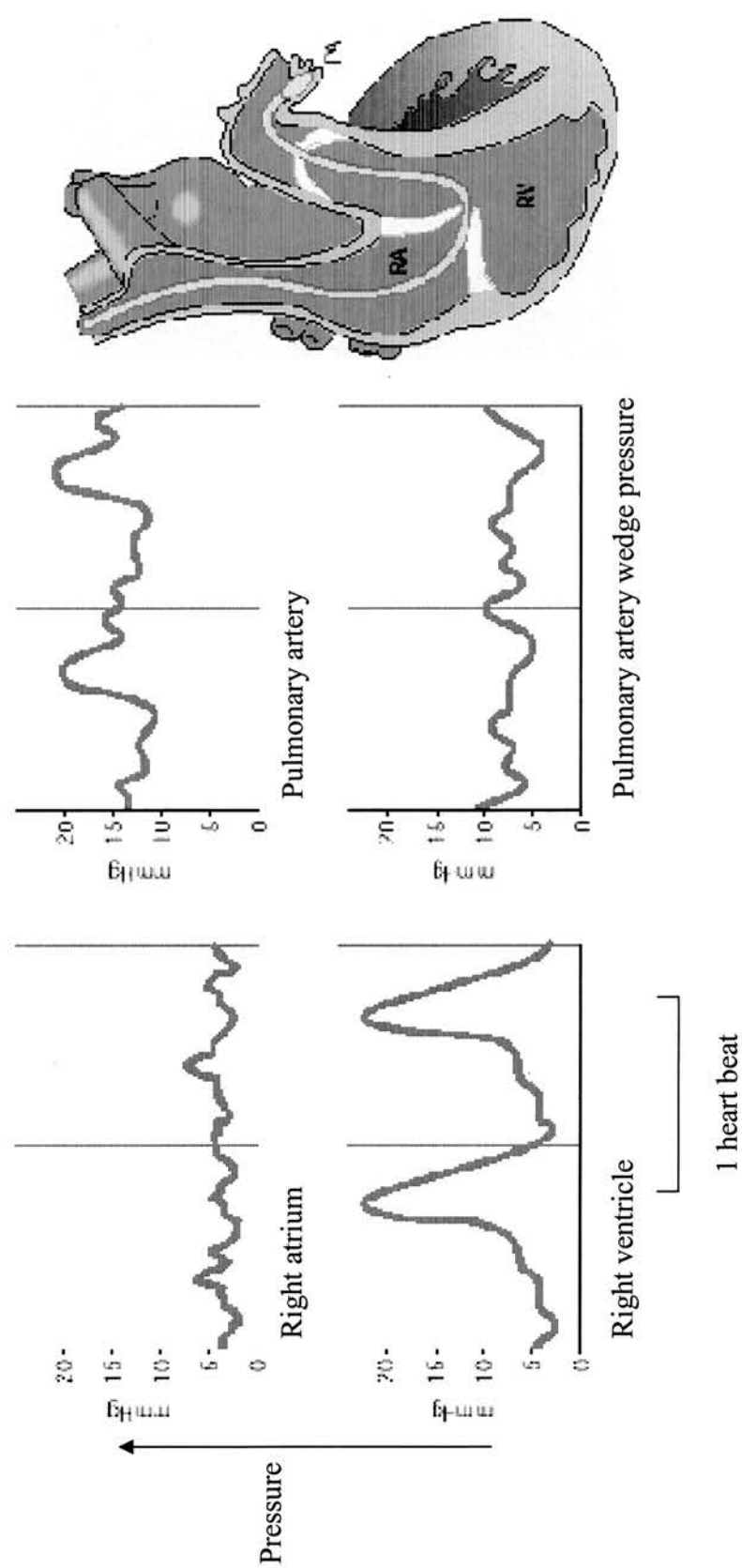
#### **2.2.1 Pulmonary artery catheter placement**

Patients were admitted to the study room (23-25 °C) and had blood pressure, pulse and electrocardiogram before being taken to the invasive procedures room of the coronary care unit. A standard clinical approach for pulmonary artery placement is usually from the subclavian or internal jugular vein. However, we found that for repeated studies insertion via the femoral vein, while technically more difficult, was well tolerated by volunteers. The patient was placed supine on the cardiology screening table and the right groin area was shaved and prepared with antiseptic solution (Betadine, Seton Healthcare

group plc, Tubitan House, Oldham, UK). The right femoral vein was cannulated with a 9F desivalve (Vygon GmbH&Co.KG, Prager Ring, Aachen, Germany) under local anaesthesia with 1% lignocaine (Antigen Pharmaceutical Ltd, Roscrea, Ireland). The pulmonary artery catheter, (Swan-Ganz CCombo - CCO/SVO2; Edwards Lifesciences LLC, Irvine, California, USA), after flushing and checking integrity of the balloon by inflating under saline, was placed into the right femoral vein and advanced to a proximal branch of the right pulmonary artery. Placement of the catheter was assisted by fluoroscopy scening and simultaneous pressure waveform monitoring (Figure 1). Adequate and consistent 'wedging' following balloon inflation was verified before the patient was transferred back to the study room.

### **2.2.2 Pressure and flow measurements**

Pressure measurements from the distal and proximal ports of the pulmonary artery catheter (PAC) were made using a cardiac computer (Horizon XL, Mennen Medical Inc. Israel). A continuous cardiac output catheter (Swan-Ganz CCombo - CCO/SVO2; Edwards Lifesciences LLC, Irvine California, USA) and computer (Vigilence, Edwards Critical Care, Baxters Healthcare Corporation, Irvine, CA, USA) were used to measure cardiac output. This catheter employs a thermistor coil to warm blood and the resultant rise in temperature can be measured distally and used to calculate 'continuous' cardiac output estimations. This avoids the need for repeated injections of cold saline thus, reducing intravenous volume loading during the study and eliminating intra-operator variability in cold injectate technique. Although termed 'continuous', measurements are taken every 30 secs.



**Figure 2.1** Pressure tracings from right atrium (RA), right ventricle (RV), pulmonary artery and pulmonary artery wedge.

### **2.2.3 Data analysis**

Data were manually recorded and entered onto a template spreadsheet (Excel v5.0; Microsoft). Recordings from the first 90 min were not used to allow stabilisation of pressures after catheter placement. Absolute change, percentage change from baseline and area under the percentage change from baseline curve were calculated.

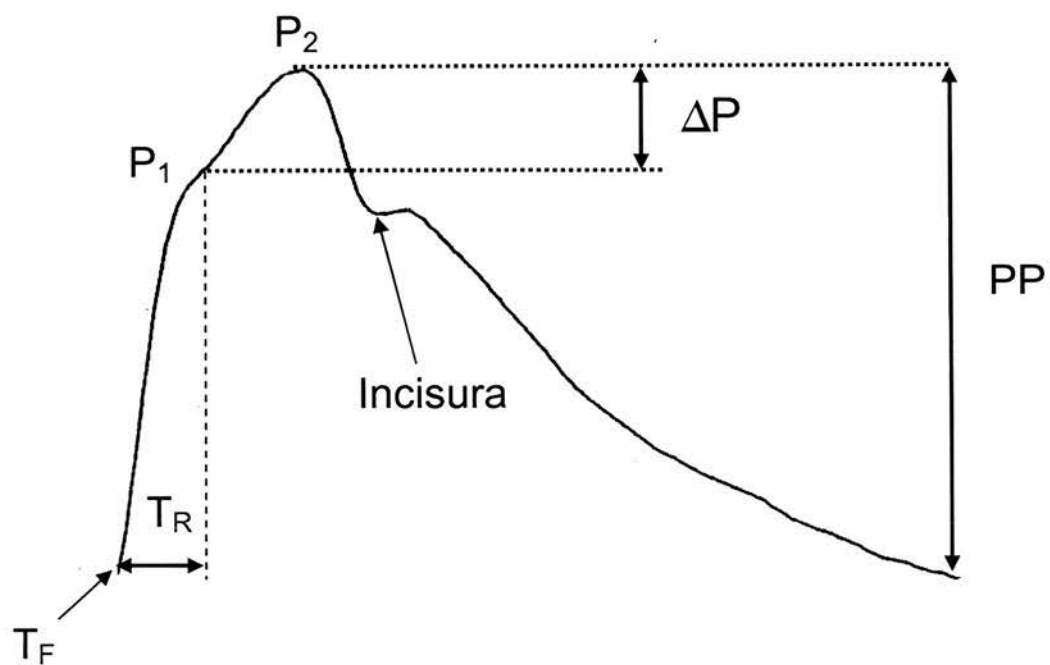
## **2.3 PULSE WAVE ANALYSIS**

### **2.3.1 Placement of tonometer**

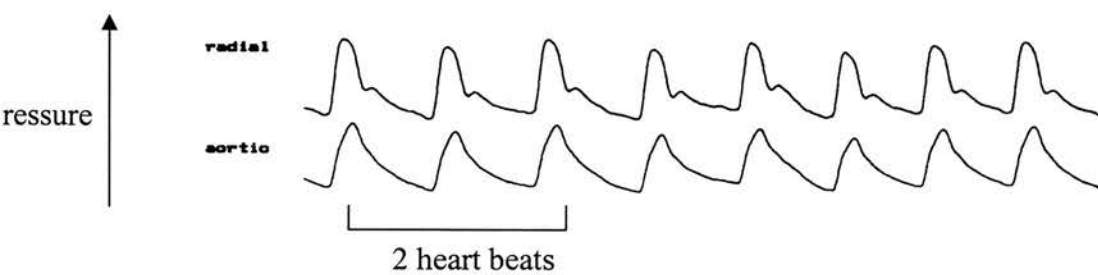
The development of pulse wave analysis (PWA) is expanding our knowledge of the stiffness of the arterial tree and its relevance to pathophysiological processes involved in the development of cardiovascular disease [O'Rourke *et al* 1996, Cockcroft *et al* 1997]. PWA is a non-invasive method using a high fidelity tonometer with computer analysis of the waveform. In theory, PWA can be performed on any artery, but for ease the radial pulse is usually used because the artery can be compressed against the underlying bony prominence of the radius bone.

### **2.3.2 Data analysis**

The arterial pressure wave has two main peaks, the first is caused by ventricular contraction, with a notch caused by aortic valve closure and a second peak caused by the reflection of the systolic wave from the peripheral vasculature (Figure 2.2). The stiffer the artery the faster the reflected wave will return. Thus, in young healthy subjects the reflected wave returns in diastole, augmenting coronary perfusion pressure while in older



**Figure 2.2** Diagram of normal radial pulse wave as measured by tonometer.



**Figure 2.3** Measured radial trace from pulse wave analysis tonometer and determined aortic wave form.

patients with stiffer arteries the wave returns earlier, in systole, thus further increasing cardiac work in systole.

## **2.4 FOREARM VENOUS PLETHYSMOGRAPHY**

Bilateral forearm plethysmography coupled with unilateral brachial artery drug infusion is a powerful tool in the investigation of the vascular responses to study compounds. This technique has been used safely in our department for many years [Webb 1995].

### **2.4.1 Experimental Setup**

The subject is studied in the supine position with the arms in a comfortable position above the level of the central venous pressure, thus allowing unimpeded venous emptying of the forearms. Venous obstruction is achieved by inflating cuffs around the upper arm to above venous pressure, but below arterial pressure (~40 mmHg) for 10 sec, then deflated for 5 sec. This cycle is repeated for 3 min at each study time period. The hands are excluded from the circulation by inflating cuffs around the wrists to above systolic pressure (~200 mmHg) during forearm blood flow measurements, as they have a non-linear response to occlusion compared to the forearm. Hand blood flow is predominantly through the skin and has different control mechanisms than FBF, which is predominantly through skeletal muscle [Whitney 1953, Whelan 1967]. It takes about 1 min following wrist exclusion for stable blood flow to occur. Cuffs were inflated using Hokanson E20 rapid cuff inflators (D.E. Hokanson, Incorporated, Bellevue, WA, USA)(Figure 2.3).





**Figure 2.4** Forearm Plethysmography

Mercury-in-silastic strain gauges EC4 (D.E.Hokanson, Incorporated, Bellevue, WA, USA) were applied to both forearms at the point of greatest circumference to measure the rate of expansion of the forearm during venous occlusion. Analogue voltage output from the strain gauge was processed by a MacLab<sup>®</sup> analogue-to-digital converter and Chart v3.3 software (AD Instruments, New Zealand) and recorded onto a Macintosh Classic II computer (Apple Computers Inc, Cupertino, USA). The rate of increase in circumference of the forearm is proportional to the blood flow and thus the vasoactive properties of compounds can be examined. Vasodilators will increase the forearm blood flow, measured as a steepening of the gradient of the curve of increasing forearm circumference, whereas vasoconstrictors have the opposite effect. A period of at least 30 minutes is usually required to establish steady baseline readings.

#### **2.4.2 Intra-arterial drug administration**

The brachial artery of the non-dominant arm was cannulated with a 27-G steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under local anaesthesia 1% lignocaine (Antigen Pharmaceutical Ltd, Roscrea, Ireland). The cannula was attached to a 16-G epidural catheter (Portex Ltd, Hythe, UK) and secured with sealing wax ('sticky wax' Cottrell and Co., London, UK). The catheter was perfused with 0.9% saline (Baxter Healthcare Ltd, Thetford, UK) via a 50 ml syringe in an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained at 1 mL/min in all studies. Repeated studies in the same subject were performed at no less than 1 week intervals.

### 2.4.3 Data analysis

Data from plethysmography studies were extracted from Chart<sup>TM</sup> data files into a template spreadsheet (Excel v5.0; Microsoft). The last 5 flow recording in each 3 minute measurement period were selected and averaged for each arm. The dominant arm acts as a contemporaneous control for the infused arm [Benjamin *et al* 1995].

## **2.5 SKIN MICROCIRCULATION FLOWMETRY**

The effects of compounds on the skin microcirculation can be investigated using intra-dermal injection of study compound and a laser Doppler flowmeter [Nilsson *et al* 1980].

### **2.5.1 Experimental Setup**

The subject was studied supine, with the arms supported and the volar aspect of the forearm upwards (Figure 2.5). The laser probe holder was attached to the skin with adhesive tape to ensure no movement of the probe throughout the study. Care was taken to avoid underlying veins which can create considerable 'noise' in the system. The subject was allowed to stabilise for at least 30 mins before the study protocol was started.

### **2.5.2 Laser Doppler flowmetry**

The laser Doppler flowmeter employs the concept of Doppler shift. Light hitting a moving object will be reflected with a change in its wavelength. The wavelength will be shortened if it is reflected from an object moving towards it and vice versa. The laser Doppler flowmeter will therefore give an estimation not only of the speed of movement of red blood cells but also their number, this combination is termed 'flux' and is proportional to blood flow.

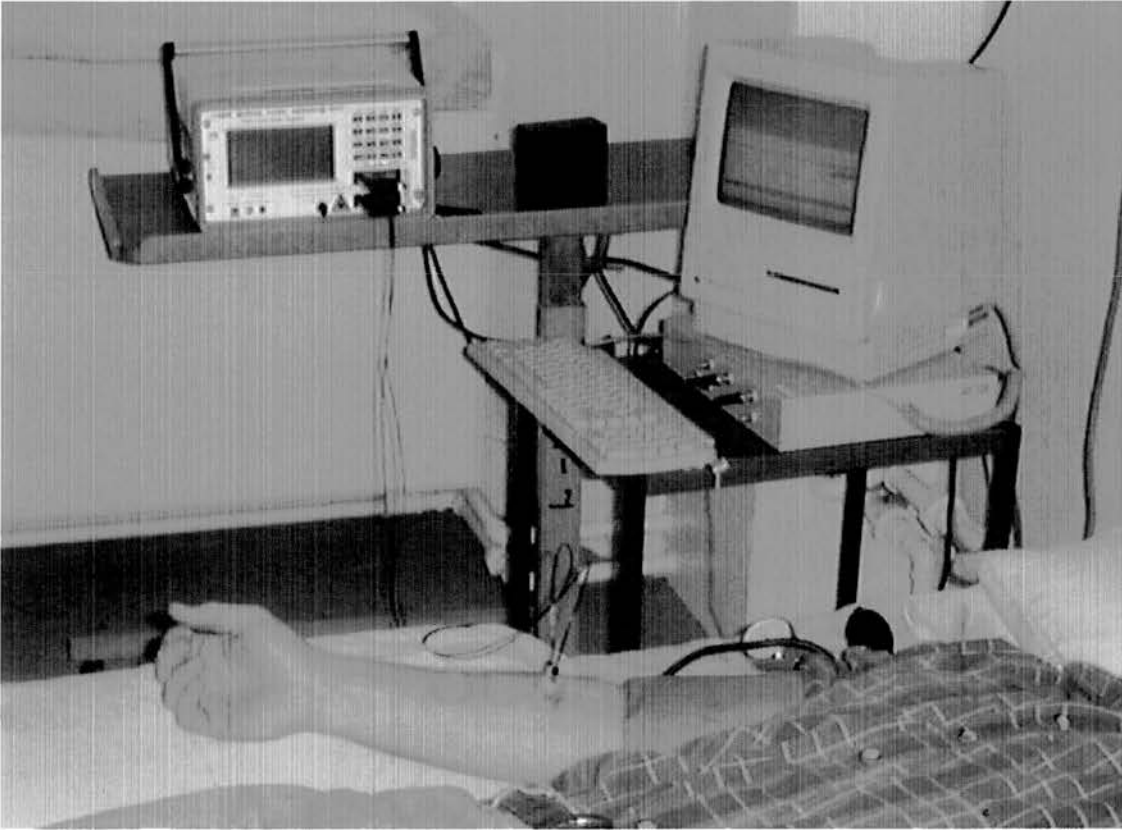
### **2.5.3 Intra-dermal drug administration**

Study compounds are injected intra-dermally using a 1ml syringe with a 0.33mm needle (Becton-Dickson, Ireland). Any injection site which did not give a characteristic weal

was rejected. The volume of injectate was small (10 $\mu$ l) and the repeatability of this technique has been assessed and shown to be highly repeatable (Chapter 3). The injection of this small volume was well tolerated by subjects. This technique uses very small doses of study compound and therefore has a high degree of safety.

#### **2.5.4 Data analysis**

Analogue voltage from the laser Doppler flowmeter was processed by a MacLab analogue-to-digital converter and Chart<sup>TM</sup> v3.3.8 software (AD Instruments Ltd, New Zealand) and recorded onto a personal computer (Classic II, Apple Computer Inc). Calibration was achieved by use of a standard flux solution (Moor Instruments Ltd, UK). 20 seconds of averaged signal were extracted from the Chart<sup>TM</sup> data files to a template spreadsheet (Excel v5.0; Microsoft).



**Figure 2.5**    Experimental setup for measurement of skin blood flow

## **2.6 ISOLATED MYOCARDIAL TRABECULAE**

### **2.6.1 Sample collection and preparation**

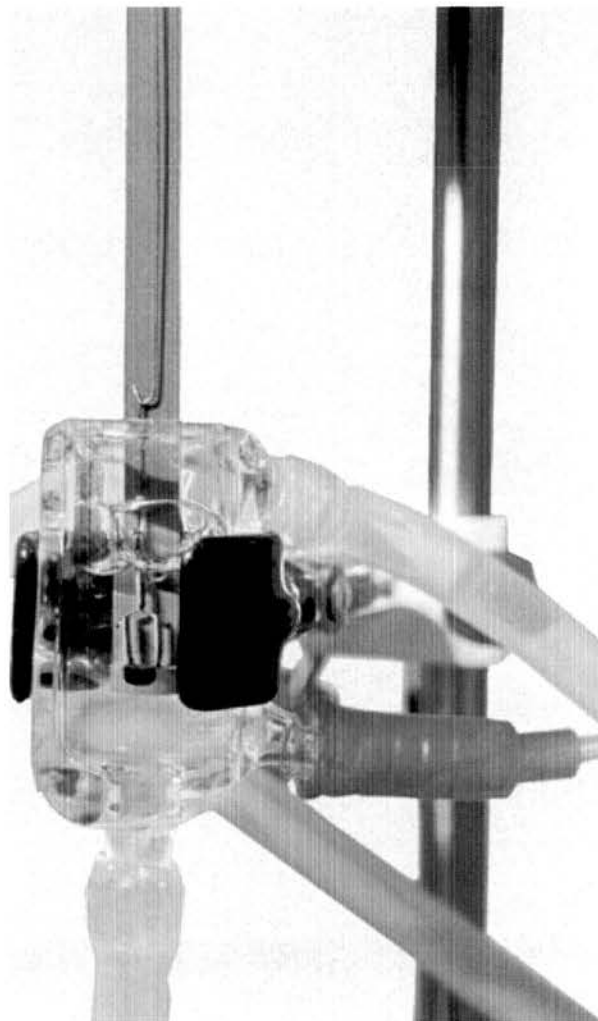
During the initiation of cardiopulmonary bypass the right atrium is cannulated to direct circulating blood from the patient to the bypass device. At the time of placement of the right atrial cannulation, while the heart is still beating, the right atrial appendage was excised and immediately placed in cold cardioplegic solution (Chapter 8). The appendage was immediately transferred to the laboratory where free running trabeculae (length < 4 mm; width < 1mm) were dissected.

### **2.6.2 Experimental apparatus**

Trabeculae were mounted for isometric tension measurement in a vertical 7ml chamber (Myobath, World Precision Instruments, Stevenage, UK) (Figure 2.6) containing physiological solution (mM); NaCl 130, KCl 5.4, NaHPO<sub>4</sub> 0.56, MgCl<sub>6</sub>H<sub>2</sub>O 3.5, CaCl<sub>2</sub>, Glucose 10 HEPES 5 and corrected to pH 7.4 with NaOH. This was continuously bubbled with 100% O<sub>2</sub> at 35°C. The lower end of the muscle was secured by a clip and the upper end tied with 5.0 silk and attached to a force transducer (Fort 10, World Precision Instruments, Stevenage, UK). The preparation was electrically stimulated with platinum point stimulation electrodes at 1 Hz at a voltage ~ 10% above threshold with rectangular pulses of 10 ms duration.

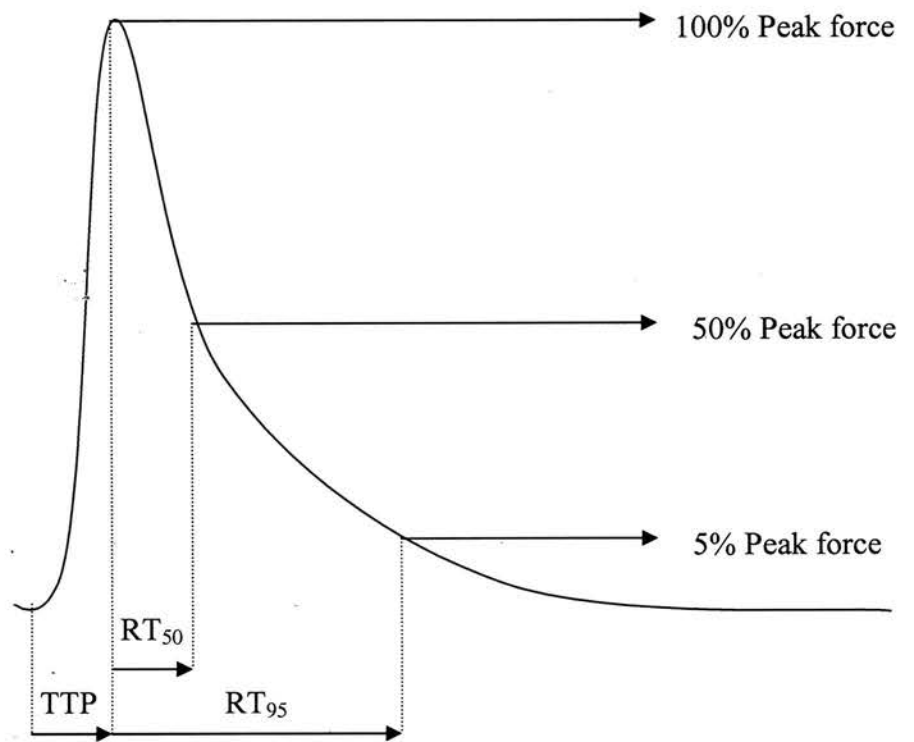
### **2.6.3 Data analysis**

Isometric twitches were detected by a force transducer (FORT 10, World Precision Instruments, Stevenage, UK), connected to an amplifier (Transbridge™) and recorded



**Figure 2.6** Myobath 7 ml chamber with atrial myocardial trabecula.





**Figure 2.7** Twitch parameters. Time to peak (TTP), time to 50% relaxation (RT<sub>50</sub>), time to 95% relaxation (RT<sub>95</sub>)

on a Power Macintosh 7200/90 personal computer using the MacLab<sup>TM</sup> analogue-to-digital converter (AD Instruments, New Zealand). The force of contraction and twitch parameters were analysed using the software 'CHART' and ink and paper traces from a chart recorder. Statistical analyses were performed using the software 'Excel'.

## **2.7 BLOOD PRESSURE, HEART RATE AND OXYGEN OXIMETRY**

Mean arterial pressure (MAP) and heart rate (HR) in the forearm plethysmography and skin microcirculation studies were measured using a validated semi-automatic digital blood pressure monitor (Omron Hem-705, Omron, Matsusaka, Japan)[O'Brien 1996]. In the systemic haemodynamic studies MAP, HR and pulse oximetry were measured using Dynamap<sup>TM</sup> compact TS (CRITIKON LLC, Coronation Road, Ascot, UK).

## **2.8 DRUG PREPARATION**

All drugs were prepared in a sterile manner. Physiological 0.9% saline (Baxter Healthcare Ltd, Thetford, UK) was used to dissolve study drugs for intra-arterial, intravenous and intra-dermal injection.

## **2.9 BLOOD SAMPLING AND PLASMA ASSAYS**

Venous blood samples for all hormone assay were collected into EDTA tubes (Sarstedt, Aktiengesellschaft & Co, Germany) on ice and immediately separated by centrifugation (2,500 rpm for 20 minutes at 4 °C). The resultant plasma was carefully pipetted into vials which were frozen and stored at -80°C until analysed. Most of the plasma assays were performed by Mr Neil Johnston.

### **2.9.1 Plasma ET-1, big ET-1 and ET-1 [1-31] extraction**

At the time of analysis, plasma samples were thawed and immediately acidified with an equal volume of 20% acetic acid. This acidified plasma sample was extracted using Bond Elut C18 columns (Varian, Harbor City, CA, USA) which had been activated with methanol, followed by washing with deionised water and 10% acetic acid. After the addition of samples to the columns, the columns were washed with 10% acetic acid and ethyl acetate, the bound ET-1 [1-31] was eluted with 80% methanol: 20% ammonium bicarbonate. The eluate was dried down under a continuous stream of nitrogen at 37°C and the dried eluates reconstituted with assay buffer.

### **2.9.2 Endothelin -1 assay**

At the time of analysis, plasma samples were thawed, immediately acidified with an equal volume of 20% acetic acid. This acidified plasma sample was extracted using Bond Elut C18 columns (Varian, Harbor City, CA, USA) that had been activated with methanol, followed by washing with deionised water and 10% acetic acid. After the addition of samples to the columns, the columns were washed with 10% acetic acid and ethyl acetate, the bound ET-1 and big ET-1 was eluted with 80% methanol: 20% ammonium bicarbonate. The eluate was dried down under a continuous stream of nitrogen at 37°C and the dried eluates reconstituted with assay buffer. Recovery of endothelin-1 and big endothelin-1 during extraction was 89% and 91%, respectively, as assessed by calculating recovery of known amounts of endothelin-1 and big endothelin-1, which had been added to plasma. Radioimmunoassay was performed on reconstituted

samples by using rabbit anti-human ET-1 or big ET-1 (Peninsula Laboratories, Europe). Briefly, 100µl of sample extract, standard or quality control along with 100µl of either ET-1 or big ET-1 antibody was incubated for 24 hours at 4°C. Following incubation, I<sup>132</sup>-labelled ET-1 (NEN Life Science Products, Boston, MA, USA) or big ET-1 (Peninsula Laboratories, Europe) was added and incubation was continued for an additional 20 hours at 4°C. The immune complexes formed were precipitated with Amerlex donkey anti-rabbit antibody (Amersham) and the precipitates counted for radioactivity. All ET values were expressed as picograms per millilitre. Intra- and inter-assay variation was 6.3 and 7.2%, respectively; sensitivity of the assay for ET-1 was 0.25pg/ml and 1pg/ml for big ET-1.

### **2.9.3 Endothelin-1<sub>[1-31]</sub> assay**

A commercially available solid phase sandwich enzyme linked immunosorbent assay for ET-1<sub>[1-31]</sub> (Immuno-Biological Laboratories Co Ltd., Tokyo, Japan) was used following the manufacturers instructions. This technique was performed on reconstituted samples by using anti-human ET – 1<sub>[25-31]</sub> Rabbit IgG. Briefly, 100µl of sample extract, standard or quality control along with 100µl of ET-1<sub>[1-31]</sub> antibody was incubated for 24 hours at 4°C. Following incubation, the wells were vigorously washed with wash buffer. 100 µl of labelled antibody was added to each well and incubated for a further 30 min at 37 °C. After further washing Tetramethyl Benzidine buffer was added, turning the solution blue, and incubated for 30 min in the dark. 100 µl of stop solution was then added, turning the solution yellow. The wells were then read using an automated plate reader at 450nm.

## **Chapter 3**

### **Validation of laser Doppler flowmetry coupled with intra-dermal injection for investigating effects of vasoactive agents on the skin microcirculation in man**

**Leslie SJ**, Affolter J, Denvir MA, Webb DJ. Validation of laser Doppler flowmetry coupled with intra-dermal injection for investigating effects of vasoactive agents on the skin microcirculation in man. *Eur J Clin Pharmacol* 2003; **59**: 99-102.

### 3.1 SUMMARY

This study was designed to determine the repeatability of laser Doppler flowmetry coupled with intra-dermal saline delivery. Two operators each performed 100 injections. Delivery of saline was judged 'by eye' using a graduated syringe (Becton-Dickinson) by injecting onto a weighing boat. Volume was assessed by weight. Skin blood flow following intra-dermal injection of saline was assessed in 18 healthy volunteers; 10 attended twice to assess between-day repeatability, and 8 attended once to assess between-site repeatability. There was no difference in mean injection weight between operators (both being  $10.3 \pm 0.1\text{mg}$ ,  $n=100$ ,  $p=0.9$ ). Intra-dermal delivery of saline was well tolerated with only mild discomfort experienced during the injection at some of the sites. Intra-dermal saline caused a 9 fold increase in blood flow ( $0.03 \pm 0.003$  to  $0.27 \pm 0.02$  perfusion units,  $n=18$ ,  $p<0.001$ ). This response was rapid in onset with the maximal effect seen at 4 min and apparent duration of greater than 30 min. There was no difference in the magnitude of the response between the dominant and non-dominant arms (AUC:  $2.9 \pm 0.4$  vs  $2.9 \pm 0.4$  respectively,  $n=18$ ,  $p=0.93$ ). However, there was no statistical difference between study visits 1 and 2 (AUC:  $3.2 \pm 0.6$  vs  $2.0 \pm 0.5$  respectively,  $n=10$ ,  $p=0.7$ ). There was no difference in the magnitude of responses between different sites on the forearm ( $p=0.6$ ). These studies demonstrate that the technique of laser Doppler flowmetry coupled with intra-dermal injection is a safe, well-tolerated technique with good repeatability. A trend towards reduced between-day repeatability emphasises the importance of vehicle control sites when investigating the effects of vasoactive compounds. This technique provides a reliable method for the intra-dermal delivery of drugs, despite the direct effect of injection of saline on blood flow.

### 3.2 BACKGROUND

Laser Doppler flowmetry is a well validated technique [Nilsson *et al* 1980] used for the investigation of the effects of vasoactive substances on the skin microcirculation [Hovell *et al* 1987, Haynes *et al* 1991, Wenzel *et al* 1994,1996 and 1998a]. Saline is widely used as a vehicle for drug administration in other vascular beds and it has been previously demonstrated that intra-dermal injection of saline, when used alone, causes an increase in laser Doppler flowmeter signal [Wenzel *et al* 1998a].

Laser Doppler flowmetry has several potential advantages over other techniques for the initial study of drugs in humans in that it is minimally invasive and relatively safe because it uses very small doses of study compounds with a mainly local action. It allows separate sites on the skin to be studied simultaneously and thus the investigation of a range of concentrations on the same occasion. While it is possible to deliver some vasoactive substances, such as acetylcholine and noradrenaline, by skin iontophoresis, many peptides cannot be delivered by this means due to their large size, poor solubility or lack of electrical charge. In these situations, intra-dermal injection can be used. However, this technique has the theoretical disadvantage that it results in a small degree of skin trauma [Holloway *et al* 1980] and delivery of the small volumes used may increase errors and variability in responses. Physiological saline is commonly used in studies as a drug vehicle and there are a variety of high precision syringes that can be used for intra-dermal injection. However, these are expensive and, when used in human studies can, by necessity, be used only for a single subject. In the current studies we used standard clinical insulin syringes with a 29.5 SWG gauge needle to administer local intra-dermal injections. The advantages

of these syringes are that they are relatively inexpensive and easy to use. These syringes have been used by other groups in skin blood flow studies [Wenzel *et al* 1998a] but have not been validated for use for intra-dermal injection in pharmacological studies. There are potential sources of error in the injection technique because the plunger is depressed by only 1 mm to deliver 10  $\mu$ l and is judged 'by eye'. The repeatability of intra-dermal delivery in terms of volume delivered and the effect of intra-dermal saline injection on skin blood flow using these syringes has not previously been reported.



### **3.3 AIMS**

The aim of these studies was to determine the intra- and inter-operator repeatability of saline administration in terms of injection volume, and to determine repeatability of skin blood flow (SBF) responses to intra-dermal injection of saline.

## 3.4 METHODS

### 3.4.1 Subjects

Eighteen, right handed, healthy men (22 - 45 years) were studied. Studies were performed with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki of the World Medical Association. Written informed consent was obtained from each subject before entry to the study. None of the subjects were taking regular medication and all avoided any medication for 1 week prior to study. All subjects abstained from alcohol for 24 hours and from food, tobacco and caffeine containing drinks for at least 12 hours before each study.

### 3.4.2 Injection volume

Graduated 29.5 SWG syringes (Becton-Dickinson, Dublin, Ireland) were used for saline delivery. Each 1 mm graduation on the 0.5 ml syringe represents 10  $\mu$ l, thus to deliver this volume the syringe plunger was depressed by 1 mm. The repeatability of injection volume was assessed by injecting 10  $\mu$ l saline judged by depressing the syringe plunger by one graduation, onto a weighing boat placed on a balance (Mettler Toledo<sup>®</sup> MT5). This balance has an accuracy and precision of 1  $\mu$ g and therefore can measure changes of 1 nl assuming a specific gravity of saline of 1.00. A new syringe was used for each injection and care was taken, as in our clinical studies, to expel any air bubbles from the syringe before injecting. The balance was set to zero before each injection. Two blinded operators each performed 100 injections.

### **3.4.3 Laser Doppler flowmetry and intra-dermal injection**

Subjects lay supine with the arms supported and the volar aspect of the forearm facing upwards. Sites for injection were chosen taking care to avoid underlying arteries (assessed by palpation and pulsatile Doppler signal) or veins (assessed visually and by high baseline Doppler signal). Four laser probe holders were attached to the skin of each forearm with adhesive tape to ensure no displacement of the probe during the study.

### **3.4.4 Protocol**

Studies were performed in a quiet, temperature controlled, draught free room (23 - 25 °C). Each subject was allowed to rest for at least 20 min before the study protocol was started. Baseline recordings were made. Subjects then received a number of intra-dermal injections of 10 µl saline (0.9 %; Baxter Healthcare Ltd, Thetford, UK) and laser Doppler signal recorded every 2 min for the first 10 min and subsequently every 5 min until 30 min. To assess between-day repeatability, 10 subjects attended for 2 study visits. To assess between-site repeatability, 8 of the subjects received 4 intra-dermal injections of saline on the volar aspect of each forearm.

### **3.4.5 Data handling and statistical analysis**

Increases in weight on the balance following saline injection were recorded manually and entered onto a spreadsheet (Excel v5.0; Microsoft). The accuracy was assessed by mean values and repeatability by assessing the spread of results. Results are expressed as mean  $\pm$  SEM. Differences between results were assessed using 2 tailed, paired Student's *t*-test. Statistical significance was taken at the 5% level.

For the skin blood flow studies, analogue voltage output from the laser Doppler flowmeter was processed by a MacLab<sup>®</sup> analogue-to-digital converter and Chart v3.3 software (AD Instruments, Castle Hill, Australia) and recorded onto a Macintosh Classic II computer (Apple Computers Inc, Cupertino, USA). Further analysis was performed off line. The average signal for 30 sec at each time point was recorded and entered into a spreadsheet (Excel v5.0; Microsoft). Area under the curve (AUC) over 30 min was calculated for each skin blood flow response curve and used to determine differences between them and expressed in arbitrary perfusion units (PU). These were assessed using the method of Bland and Altman [Bland *et al* 1986]. Bland-Altman analysis allows the assessment for agreement and systematic bias. Coefficients of repeatability were determined for 95% confidence intervals (CI). Other results are expressed as mean  $\pm$  SEM. Statistical analysis was performed using 2 tailed, paired Student's *t*-test and single factor ANOVA for between-site repeatability. Statistical significance was taken at the 5% level.

## **3.5 RESULTS**

### **3.5.1 Accuracy and Repeatability of Injection Volume**

Two operators performed 100 injections each. There was no difference in mean injection weight between operators, both being  $10.3 \pm 0.1\text{mg}$  [0.08, -0.23 to 0.39 mg: mean difference, 95% confidence interval,  $n=100$ ,  $p=0.9$ ] (Figure 3.1).

### **3.5.2 Tolerability of intra-dermal drug delivery**

The technique was well tolerated by subjects with only mild discomfort experienced during the injection of saline at some of the sites. This discomfort was variable in intensity. In the majority subjects the trauma from intra-dermal injection did not leave any discernable mark on the skin by the end of the study although several injections did cause a small degree of bleeding along the track of the needle. This did not appear to affect the results in terms of repeatability.

### **3.5.3 Skin Blood Flow Responses:**

#### *3.5.3.1 Effect of intra-dermal injection of saline*

Saline caused a 9 fold increase in skin blood flow [ $0.03 \pm 0.003$  to  $0.27 \pm 0.02$  PU,  $n=18$ ,  $p<0.001$ ]. This effect was rapid in onset with maximal response seen at 4 min (Figure 3.2)

#### *3.5.3.2 Between-site repeatability*

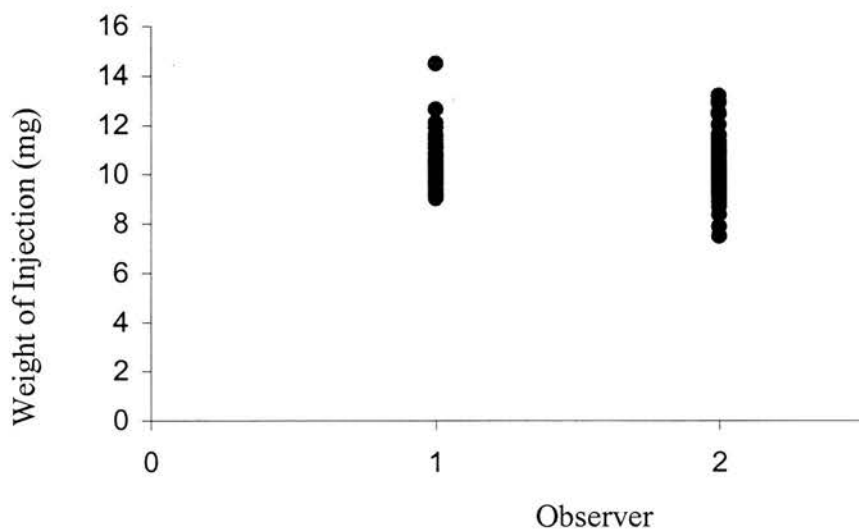
Area under the curve was constructed for the responses at 4 different sites on each forearm in 8 subjects. There was no difference in the magnitude of responses between sites on the forearm as assessed by AUC ( $p=0.6$ )(Figure 3.3).

#### 3.5.3.3 *Between-arm repeatability*

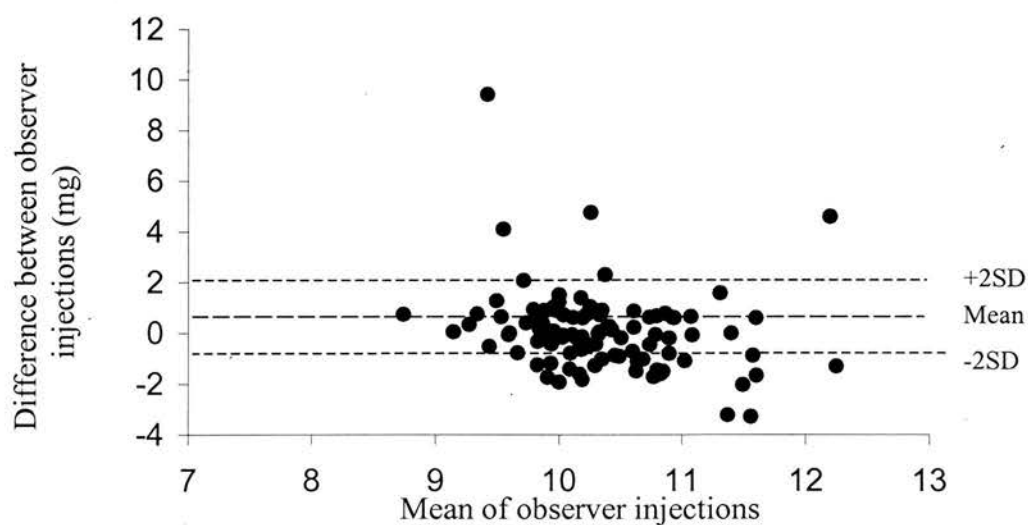
There was no difference in the magnitude of the response between the dominant and non-dominant arms, AUC was  $2.9 \pm 0.4$  and  $2.9 \pm 0.4$  respectively [-0.05, -0.8 to 0.73 PU: mean difference and 95% CI, n=18, p=0.93] (Figure 3.4a). Bland-Altman analysis was performed demonstrating no systematic bias and a co-efficient of repeatability of 3.54 (Figure 3.4b).

#### 3.5.3.4 *Between-day repeatability*

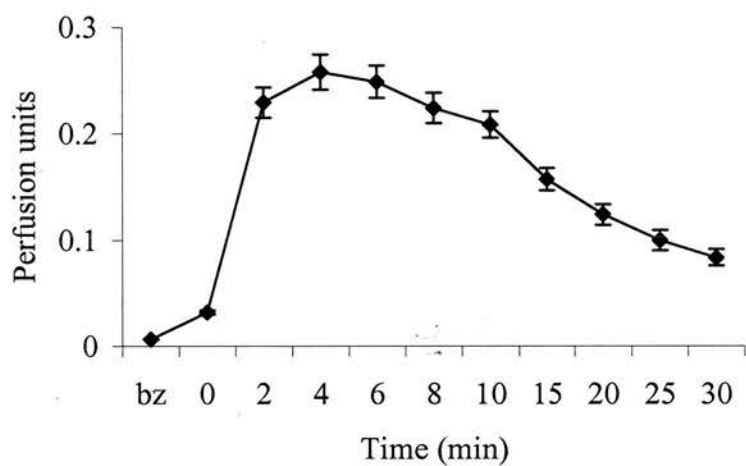
There was a trend towards a difference between study visits 1 and 2, AUC was  $3.2 \pm 0.6$  and  $2.0 \pm 0.5$  respectively [1.2, -0.03 to 2.43 PU: mean difference and 95% CI n=10, p=0.7] (Figure 3.5a). Bland-Altman analysis was performed and tended to show systematic bias and a co-efficient of repeatability of 3.48 (Figure 3.5b).



**Figure 3.1a** Scatter plot of injection weights demonstrating spread of results. Both observers had the same mean value and standard deviation ( $10.3 \pm 0.1$ ).

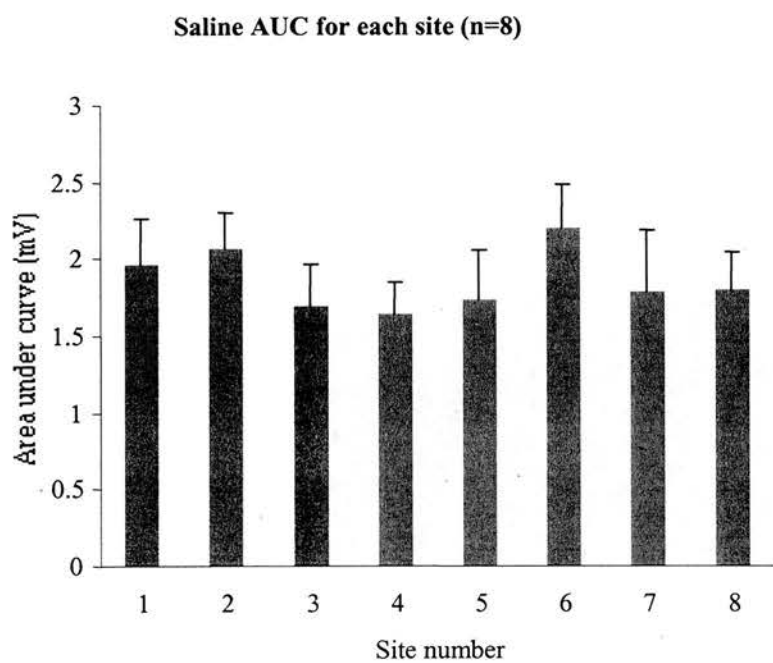


**Figure 3.1b** Bland – Altman plot of injection volumes by 2 observers demonstrating good repeatability of results. The confidence limits are marked at mean difference  $\pm 2SD$ .

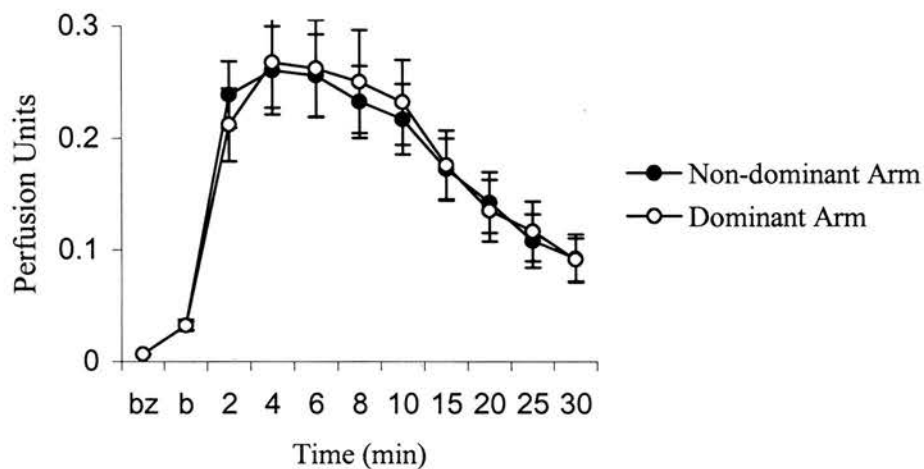


**Figure 3.2** Effect of skin blood flow in response to intra-arterial saline (0.9%). This graph represent all measurement for studies 2 and 3. Biological zero (bz).

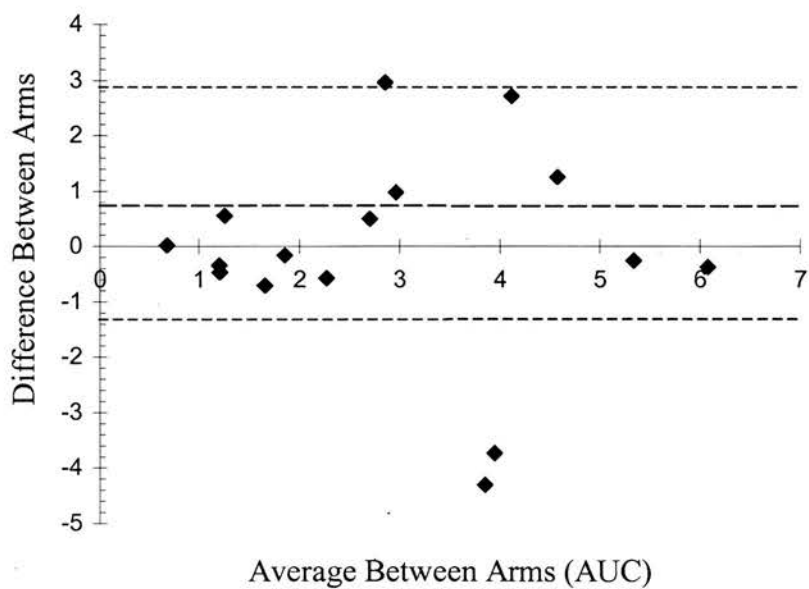




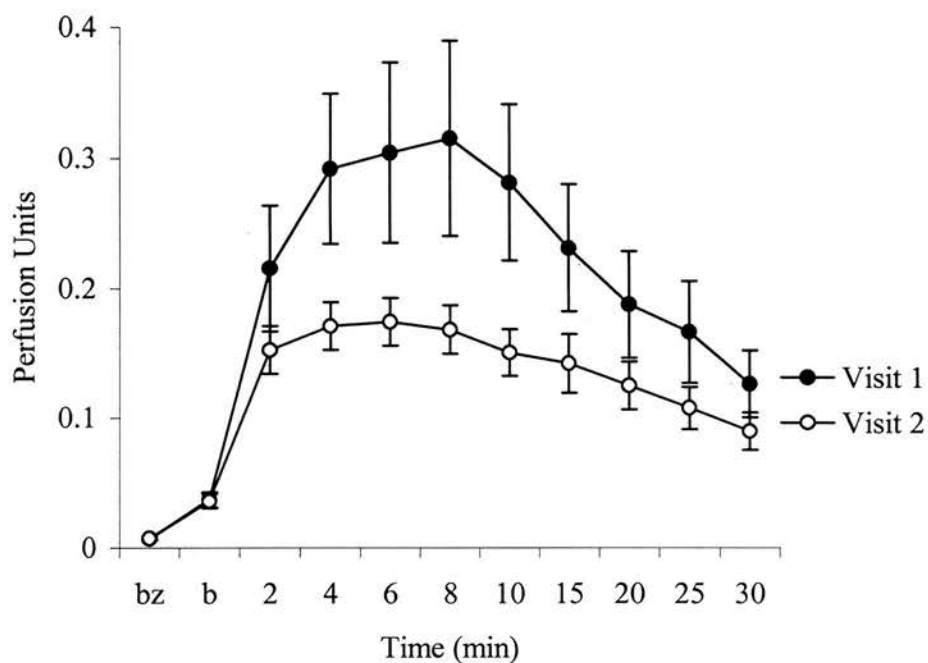
**Figure 3.3** Between site variation in skin blood flow (p=0.6, single factor ANOVA)



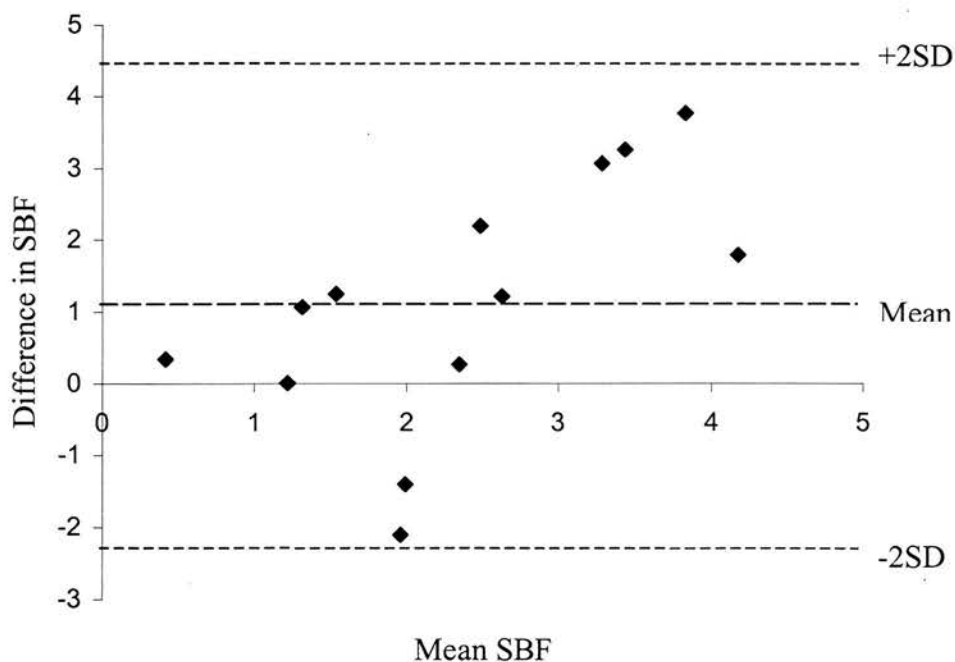
**Figure 3.4a** Skin blood flow response to intra-dermal saline ( $p = 0.93$ , single factor ANOVA)



**Figure 3.4b** Bland-Altman plot of skin blood flow between dominant and non-dominant arms



**Figure 3.5a** Skin blood flow responses to intra-dermal saline between visit 1 and 2  
Mean  $\pm$  SEM ( $p=0.7$ , single factor ANOVA)



**Figure 3.5b** Bland-Altman for between-day repeatability

### 3.6 DISCUSSION

In this study we have demonstrated that saline delivery using the Becton-Dickinson syringe is accurate and repeatable with low intra-operator variability. In addition, we have demonstrated that intra-dermal injection of saline causes a clear increase in Doppler signal but that the magnitude of this increase is similar between different sites on the forearm. There is good within-day and between arm repeatability.

There was occasionally mild discomfort, of variable intensity, experienced by the subjects at the time of injection. However, there did not appear to be any pattern to explain the fact that some sites developed more pain than others. Although the non-uniform distribution of cutaneous nerves may explain this finding, this was not formally assessed but did not appear to affect the results in terms of repeatability.

While there is often greater interest in the systemic effects of vasoactive compounds, there are potential risks with administration of systemic doses of vasoactive compounds. The use of local techniques, such as intra-dermal administration with laser Doppler microcirculatory blood flow measurement has allowed the relatively safe observation of the *in vivo* effects of vasoactive compounds, without causing confounding compensatory systemic effects. While the skin blood flow may be under different mechanisms of control than other vascular beds, and responses can differ [Weber *et al* 1997], in general, effects of compounds in the skin have been reflected in other less accessible vascular beds [Wenzel *et al* 1996 and 1998a]. Therefore, the skin microcirculation offers a safe, well tolerated, easy to use approach to the initial investigation of vasoactive compounds.

The Becton-Dickinson syringe is commonly used to delivery intra-dermal injections in clinical practice. Although it has the advantage that it is inexpensive and easy to use, its accuracy has not previously been described. Here, we found good agreement between operators as seen by mean values that were similar and close to the intended volume of 10  $\mu$ l with most injections very close to this volume (Figure 1). We conclude that this syringe can be used to deliver intra-dermal injections in a clinical research setting with sufficient accuracy and repeatability.

Intra-dermal injection of saline causes an increase in laser Doppler signal. In this study, we have demonstrated that the technique of laser Doppler flowmetry coupled with intra-dermal injection is a repeatable technique and that responses between subjects are similar. There was no difference in SBF in response to intra-dermal saline injection between sites on the same arm or in the same patient on different study visits. There was, however, a trend towards a small difference in SBF between study visits which emphasises the importance of vehicle control when investigating the effects of vasoactive compounds. The reasons for this between-day variability are not clear as ambient temperature was controlled, subjects were fasted and studied under similar conditions during each study visit. Although careful attempts were made to keep room temperature and conditions constant, the skin is more sensitive than other vascular beds to changes in ambient conditions and small temperature changes, draughts or emotional factors may be more important than in other vascular beds. Nevertheless, these between day differences did not reach statistical significance and the within-subject and between-day coefficients of repeatability

were similar (3.54 vs. 3.48). However, this finding may represent a type II statistical error as it is clearly seen on Figure 3.5a that there is a difference between results on different days. Therefore despite no statistical difference noted between days we believe that vehicle injections should be performed as controls during each study visit.

In conclusion, the technique of intra-dermal drug delivery with saline vehicle coupled with laser Doppler flowmetry to measure skin microcirculatory blood flow offers a safe, well-tolerated, repeatable technique for the investigation of vasoactive compounds in human *in vivo*.

## **Chapter 4**

### **Comparison of two plethysmography systems in assessment of forearm blood flow**

**Leslie SJ**, Attina T, Hultsch E, Bolscher L, Grossman M, Denvir MA, Webb DJ. Comparison of two forearm plethysmography systems, in assessment of forearm blood flow. *J Appl Physiol* 2004;96:1794-1799.

#### 4.1 SUMMARY

Venous occlusion plethysmography is widely used to assess forearm blood flow (FBF). Recently a new device has been developed, we compared the established Hokanson (HEC4) system with the Filtrass 2001 (F2001) to determine if this new device would offer significant advantages in future studies. The HEC4 uses 'mercury-in-silastic' strain gauges while F2001 detects volume changes using a non-mercury linear displacement device. The aim of this study was to evaluate the new F2001 against the HEC4 in terms of repeatability and systematic bias. 10 subjects were studied on 4 separate days in random order using either the HEC4 on both arms (H/H), the F2001 on both arms (F/F), the HEC4 on the right arm with the F2001 on the left (H/F) or the F2001 on the right arm and the HEC4 on the left (F/H). Stroop's coloured word conflict test (SCWT) and post-occlusive hyperaemia were used to increase FBF and lower body negative pressure (LBNP) was used to lower FBF. SCWT and LBNP increased ( $24.6 \pm 1.5\%$ ,  $n = 240$ ,  $P < 0.0001$ ) and decreased ( $18.7 \pm 0.8\%$ ,  $n = 240$ ,  $P < 0.0001$ ) FBF respectively. Post occlusive hyperaemia following occlusion times of 5, 8 and 13 mins substantially increased FBF by  $390 \pm 86\%$ ,  $756 \pm 217\%$  and  $851 \pm 132\%$  respectively. Repeatability was not different between the devices ( $0.10 \pm 2.37$  vs  $-0.47 \pm 1.92$  l/min,  $n = 125$ ,  $P > 0.05$ ) and there was no systematic bias. The F2001 is a newly developed plethysmography system that does not utilise mercury and which is suitable for assessing changes of FBF in physiological studies. However, there appeared to be no significant improvements in repeatability over the HEC4, which is the established device in our department, and thus the HEC4 was used in subsequent studies.



## 4.2 BACKGROUND

Venous occlusion plethysmography has been used to study forearm blood flow (FBF) for almost 100 years [Brodie *et al* 1905]. The underlying principle is simple; by obstructing venous outflow, but not arterial inflow, the forearm volume initially increases in proportion to the FBF. The standard technique for the assessment of this change in FBF, using a circumferential mercury-in-silastic strain gauge as part of a Wheatstone bridge to detect increases in forearm circumference and derive volume changes, has remained essentially unchanged for 50 years [Whitney 1953]. Venous occlusion plethysmography is now a well validated [Roberts *et al* 1986] and widely used tool to study mechanisms of human vascular control, particularly when coupled with brachial artery infusion to study effects of drugs and mediators on the forearm vasculature [Joyner *et al* 2001]. In man, this approach to vascular pharmacology has a distinct advantage over other techniques in that vessels are studied in their physiological environment [Benjamin *et al* 1995, Joyner *et al* 2001]. A commonly used and well validated device employing the above principles is the Hokanson EC4 (HEC4) [Hokanson *et al* 1975, Chang *et al* 1987, Petrie *et al* 1998, Fehling *et al* 1999, Rongen *et al* 1999, Strachan *et al* 2002].

Recently, a new device, the Filtrass 2001 (F2001) has been developed, particularly to measure capillary permeability. However, this device may also be useful for measuring FBF. It is based on similar principles, but with some important differences. Here, forearm circumferential measurement is measured by displacement of a freely moveable core of a linear variable displacement transducer

(LVDT) attached to a circumferential plastic monofilament (Fig 4.1). A linear relationship exists over a large range of calibration movements [Christ 2000]. The system is mechanically and electrically calibrated to measure differences of 10  $\mu\text{m}$  and undergoes internal calibration for changes in temperature and tissue compliance at each time point. The potential utility of this device in forearm venous occlusion plethysmography has not previously been assessed.

The aim of this study was to compare the F2001 against the established HEC4 in terms of repeatability, systematic bias and ease of use. In order to compare the F2001, subjects underwent simple, non-invasive systemic interventions to reduce or increase FBF. It was assumed that systemic haemodynamic changes would result in similar changes in FBF simultaneously in both arms [Greenfield *et al* 1954]. In order to assess higher blood flows post-occlusive hyperaemia was used. In this study the repeatability of each individual device could be assessed when the same device was used on both arms. Comparison of one system against the other on different arms allowed examination for systematic bias between devices.

### **4.3 METHODS**

#### **4.3.1 Subjects**

Ten healthy subjects (9 male) aged 22-30 years were recruited for this study, which was undertaken in accordance with the Declaration of Helsinki and with the approval of the local research ethics committee. Written informed consent was obtained from each subject. All subjects abstained from vaso-active drugs for at least 1 week, from alcohol and cigarettes for 24 hours, and from food and caffeine for at least 3 hours before each study visit.

#### **4.3.2 Study design**

Volunteers attended for 2 separate study periods, each of 4 visits. The protocol was similar on each of the first 4 study days except for the plethymography device used. The devices were studied in random order using either the HEC4 on both arms (H/H), the F2001 on both arms (F/F), the HEC4 on the right arm with the F2001 on the left arm (H/F) or the F2001 on the right arm and the HEC4 on the left arm (F/H). During the second 4 study visits, post occlusive hyperaemia was employed to cause a range of increases in FBF.

#### **4.3.3 Study protocol**

The study visits were performed on different days in a quiet, draught free, temperature controlled room (22-24 °C). During the 'low flow' experiments the volunteer was subjected to either SCWT, LBNP or a rest period. Mean arterial pressure and heart rate were measured during the 6<sup>th</sup> min, and FBF measured during

the last 3 min, of each 10 min time period. During the 'high flow' experiments volunteers were exposed to 5, 8 and 13 mins of arterial occlusion resulting in post occlusive reactive hyperaemia.

#### **4.3.4 Venous occlusion forearm plethysmography**

Both devices were employed in a similar manner using the standard methodology employed in our unit [Wilkinson & Webb 2001]. In brief, venous occlusion was achieved by inflating cuffs around the upper arm to above venous, but below arterial, pressure (~40 mmHg). The arm was placed above the level of the right atrium and the upper arm cuff inflated for 10 sec, then deflated for 5 sec. This cycle was repeated for the last 3 min of each 10 min study time period. Hand blood flow is predominantly through skin blood vessels rather than skeletal muscle and thus has different control mechanisms than FBF [Whitney 1953, Scroop *et al* 1965, Whelan *et al* 1967]. Therefore, the hands were excluded from the circulation by inflating cuffs around the wrists to above systolic pressure (~200 mmHg) before FBF measurement. The strain gauges were placed around the forearm at the point of greatest circumference.

There were some features unique to each device. The HEC4 system (D. E. Hokanson Inc, Bellevue, WA, USA) consists of several discrete components: 2 HEC4 plethysmographs, 2 Hokanson E20 rapid cuff inflators, a Hokanson AG 101 cuff inflator air resource, strain gauges, and Hokanson wrist and upper arm cuffs. The HEC4 system uses electrically calibrated mercury-in-silastic strain gauges that were

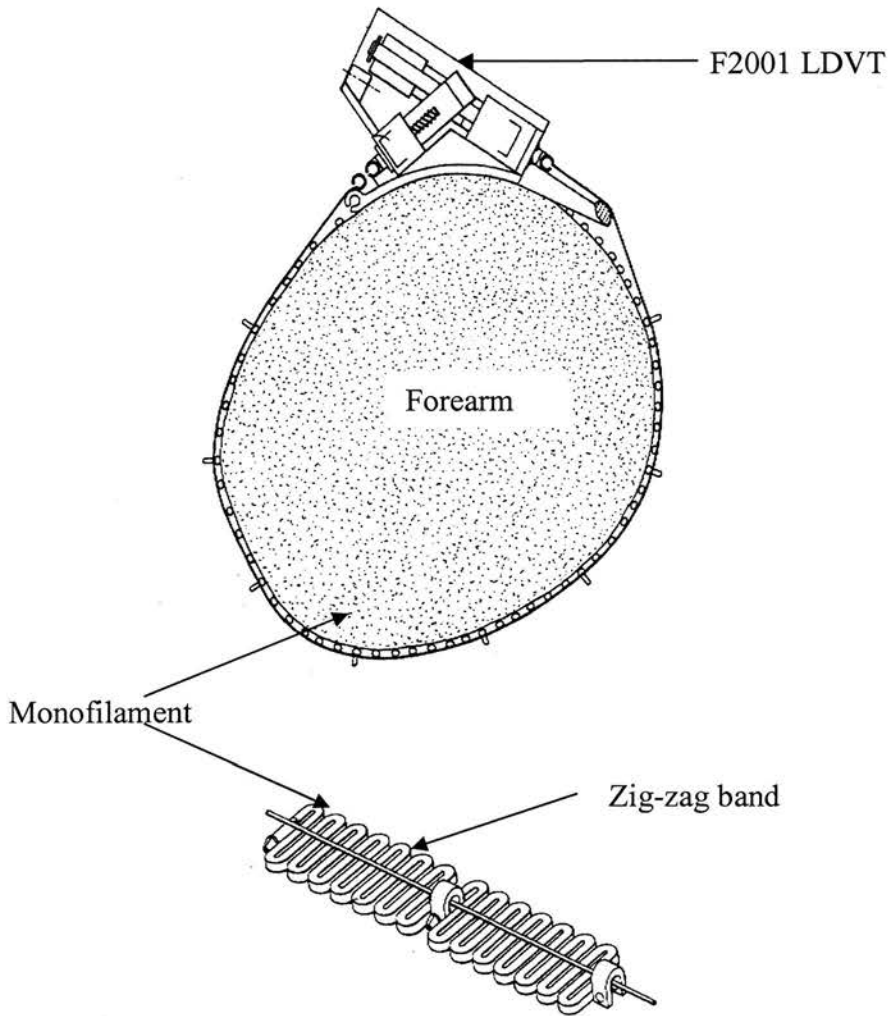
calibrated at the beginning of each study. The HEC4 occupies a total volume of  $1.4\text{m}^3$ .

The F2001 LVDT (DOMED Medizintechnik, Munich, Germany) was positioned on the forearm skin at a similar position to the mercury-in-silastic strain gauges, and fixed to the forearm with adhesive tape. The plastic monofilament component surrounding the forearm is supported on the skin by a 'zig-zag band' (Figure 4.1). Before the start of measurements, the surface of the monofilament was coated with synthetic oil (DOMED Medizintechnik, Munich, Germany) to reduce friction and then inserted into the main body of the LVDT. The LVDT was calibrated at the beginning of each study and the resting tension on the monofilament adjusted automatically at each time point. The F2001 is a smaller, more compact, device than the HEC4 with all components contained within a single housing with a volume of  $0.22\text{ m}^3$ .

#### **4.3.5 Lower body negative pressure (LBNP)**

The lower body of the subject was placed in an airtight steel chamber and sealed with a pneumatic belt around the waist (Figure 4.2). Suction was applied using a low pressure vacuum device and the negative pressure within the chamber kept constant by a servomechanical pressure regulator (Department of Medical Physics, Western General Hospital, Edinburgh, UK). LBNP (15mm Hg) was applied for 10 min to reduce FBF. LBNP causes venous pooling which, via baroreceptor unloading and selective activation of elements of the sympathetic nervous system, results in an increase in systemic vascular resistance, forearm vascular resistance and thus a

reduction in FBF. Previous studies have shown that there is no effect on mean arterial pressure or heart rate at this level of negative pressure [Laszlo *et al* 1998].



**Figure 4. 1** Filtrass 2001 plethysmography device and Filtrass 2001 'zig zag' band and linear displacement device



**Figure 4.2** Lower body negative pressure chamber.



#### **4.3.6 Stroop's coloured word conflict test (SCWT)**

The SCWT consists of several pages of the words: 'blue', 'red', 'yellow', and 'green' each printed in different colours of ink (blue, red, yellow and green) in a random order (Figure 4.4). The subject is asked to state the colour of the ink in which the word is printed not the printed word. Mental conflict arises because the learned response is to read the word rather than report the colour. This task was performed at a steady rate of ~ 100 words per min, with a metronome and observer acting as a guide to encourage volunteers to keep to this pace. In order to induce reproducible mental stress in our subjects we applied the SCWT for 10 min. Responses to the SCWT include a decrease in forearm vascular resistance and a marked increase in FBF [Lindqvist *et al* 1997]. SCWT also causes an increase in mean arterial pressure, heart rate, cardiac output and a fall in systemic vascular resistance. Increases in FBF are stable and reproducible after 9 min [Freyschuss *et al* 1988]. However, forearm vasodilator responses to SCWT may become diminished with repeated exposure [Joyner *et al* 1997] and, in order to cause a range of FBF changes, three 10 min exposures to SCWT were performed at each study visit.

#### **4.3.7 Post occlusive hyperaemia (POH)**

Brachial artery occlusion was applied by manually inflating an upper arm cuff to at least 60mmHg above systolic pressure. Post occlusive hyperaemia of the forearm resulted after the occlusion was released. A range of occlusion times (5, 8 and 13 mins) were used to assess the devices over a range of FBF.

green	red	blue	orange	purple	yellow
red	grey	purple	yellow	blue	pink
red	green	grey	blue	purple	blue
blue	pink	grey	green	red	yellow
purple	blue	red	yellow	green	green
blue	pink	yellow	blue	purple	red
green	pink	red	grey	purple	green
yellow	red	green	blue	grey	pink
blue	green	grey	purple	blue	blue
red	purple	green	yellow	pink	yellow
green	yellow	blue	red	green	grey
grey	blue	purple	blue	grey	green
red	blue	green	purple	red	purple

Figure 4.3

Stroop's coloured word test

#### **4.3.8 Data acquisition and statistical analysis**

FBF was obtained from the mean of the last 5 consecutive recordings of each period. Both systems allow the manual rejection of curves if rendered unsuitable for analysis by movement artefact. Each slope recording was taken from the steep linear part of the response curve. In the high flow experiments the plateau phase was reached more quickly but the steep linear portion of the curve was still easily identified and assessed over 3 or more heart beats. Data obtained from the Hokanson plethysmograph were stored on a Macintosh computer, using the Chart v3.3 software (MacLab, AD instruments, New Zealand). The data were further analysed off line using the Chart v3.3 software and a template spreadsheet (Excel 5.0; Microsoft). The data from the F2001 were analysed in a similar manner using a PC-based automatic analysis programme, which fits a curve to the measured slope and produces a mean value for FBF.

There is no 'gold standard' for the non-invasive measurement of FBF. Repeatability of the devices was quantified by the average difference between the changes in FBF, from the preceding rest period, in response to stimuli, measured by the same device when used simultaneously on left and right arms (H/H and F/F). Therefore, assuming stimuli will affect the FBF in each arm to a similar degree, the closer to zero the difference in FBF between each arm and the smaller the standard deviation of results, the more repeatable the device. Systematic bias between devices was determined by the average of the differences from the mean when different devices were simultaneous used to measure the changes in FBF (H/F and F/H) and presented as Bland-Altman plots [Bland 1986].

The effects of the SCWT, LBNP and post occlusive hyperaemia on FBF were assessed using all data from both devices on left and right arms and expressed as mean percentage changes from baseline  $\pm$  SEM. Mean differences were assessed using paired, 2 tailed, Student's *t*-test. Statistical significance was taken at the 5% level. The study had 80% power to show a 7.5% difference between the devices assuming a 15% standard deviation in FBF measurement.

## 4.4 RESULTS

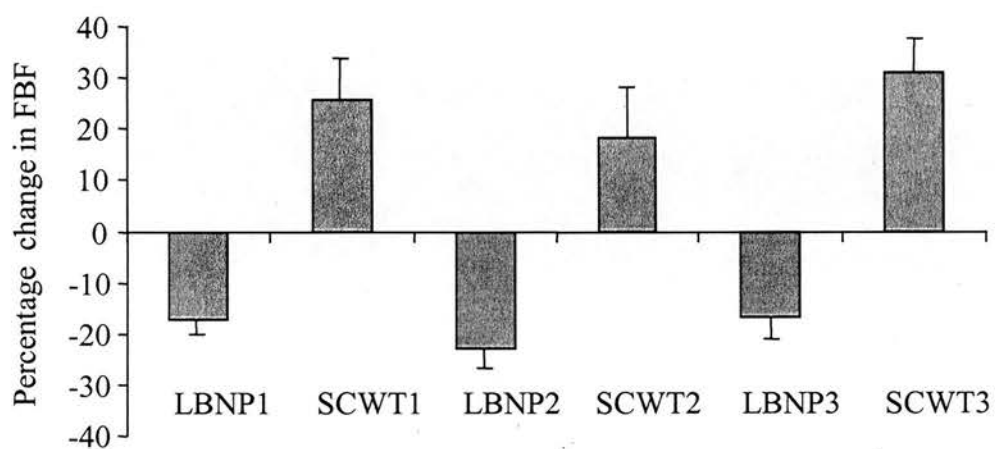
None of the subjects were obese. All were right handed with correspondingly larger right forearm circumferences ( $264 \pm 5$  vs  $259 \pm 5$  mm,  $P < 0.01$ ). However, there was no difference between baseline blood flow between the left and right arms ( $3.1 \pm 0.2$  vs  $2.6 \pm 0.1$  mL/100mL/min,  $P = 0.1$ ). The HEC4 cuff is broader than the F2001 cuff and inflates in less than 1 second whereas the F2001 cuff takes 3-4 seconds to inflate. There did not appear to be any difference in cuff 'artifact' between the two systems as a result.

LBNP and SCWT caused the expected changes in FBF, which were repeatable within study visits (Figure 4.4a). LBNP caused a decrease in FBF ( $-18.7 \pm 0.8\%$ ,  $n = 240$ ,  $P < 0.0001$ ) and SCWT caused an increase in FBF ( $24.6 \pm 1.5\%$ ,  $n = 240$ ,  $P < 0.0001$ ). Forearm hyperaemia following occlusion times of 5, 8 and 13 mins substantially increased FBF by  $390 \pm 86\%$ ,  $756 \pm 217\%$  and  $851 \pm 132\%$  respectively (all  $P < 0.0001$ )(Figure 4.4b).

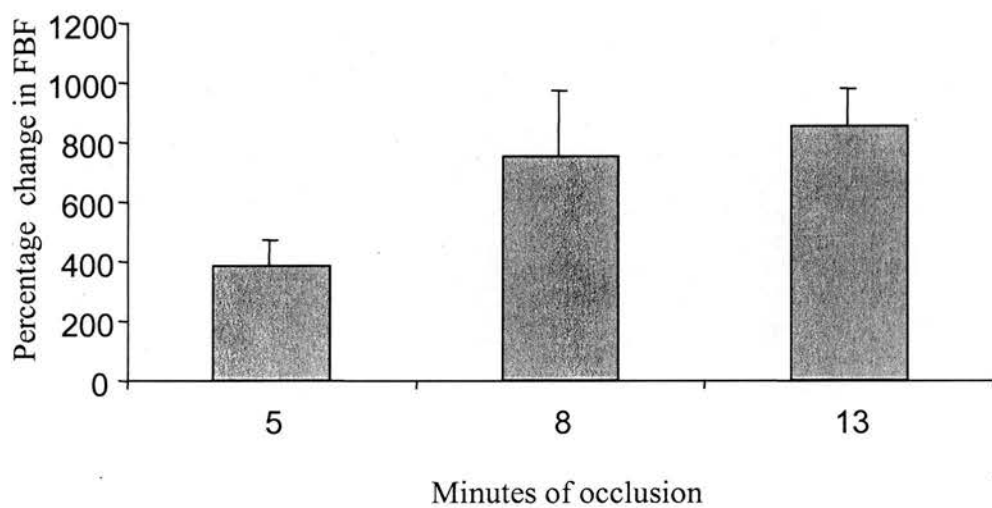
### 4.4.1 Comparison between HEC4 and F2001

When used to measure changes in the left and right arm, the HEC4 showed no difference in the absolute change in FBF between arms ( $0.10 \pm 2.37$  l/min  $n = 125$ ,  $P > 0.05$ ). When used to measure simultaneous changes in the left and right arm, the F2001 showed no difference in absolute changes in FBF between arms ( $-0.47 \pm 1.92$  l/min,  $n = 125$ ,  $P > 0.05$ ). There was no difference between the HEC4 and F2001 ( $0.10 \pm 2.37$  vs  $-0.47 \pm 1.92$  l/min,  $n = 125$ ,  $P > 0.05$ ). There was good correlation

between the devices (Figure 4.5). Bland-Altman plots reveal no systematic bias between the devices or in relation to flow values when used to measure simultaneous changes in response to the systemic stimuli described above (Figure 4.6).



a)



b)

Figure 4.4

a) Effect of lower body negative pressure (LBNP) and Stroop's colour word test (SCWT) on forearm blood flow. b) Effect of post-occlusive hyperaemia on forearm blood flow. Changes are expressed as percentage change from the preceding baseline.

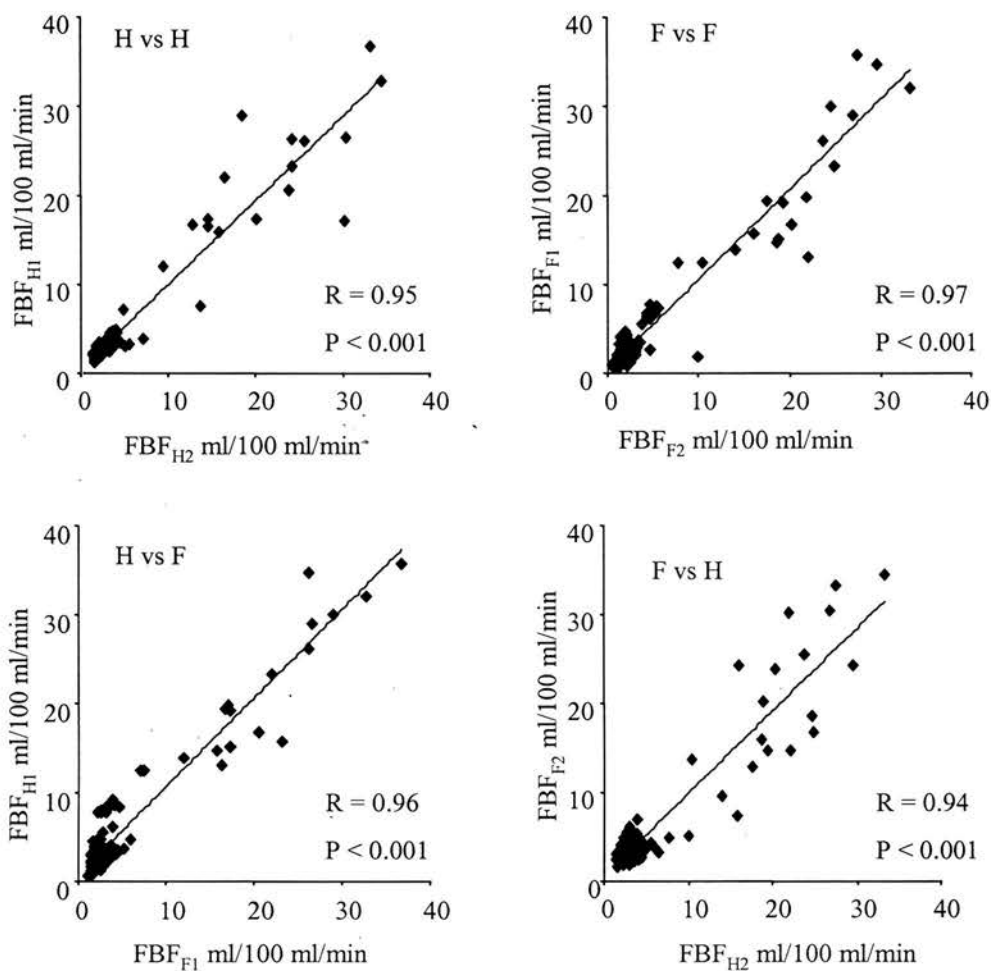


Figure 4.5

Correlation graphs of (a) Hokanson EC4 (right arm) vs Hokanson EC4 (left arm) (b) Filtrass 2001 (right arm) vs Filtrass 2001 (left arm) (c) Filtrass 2001 (right arm) vs Hokanson EC4 (left arm) (d) Hokanson EC4 (right arm) vs Filtrass 2001 (left arm).



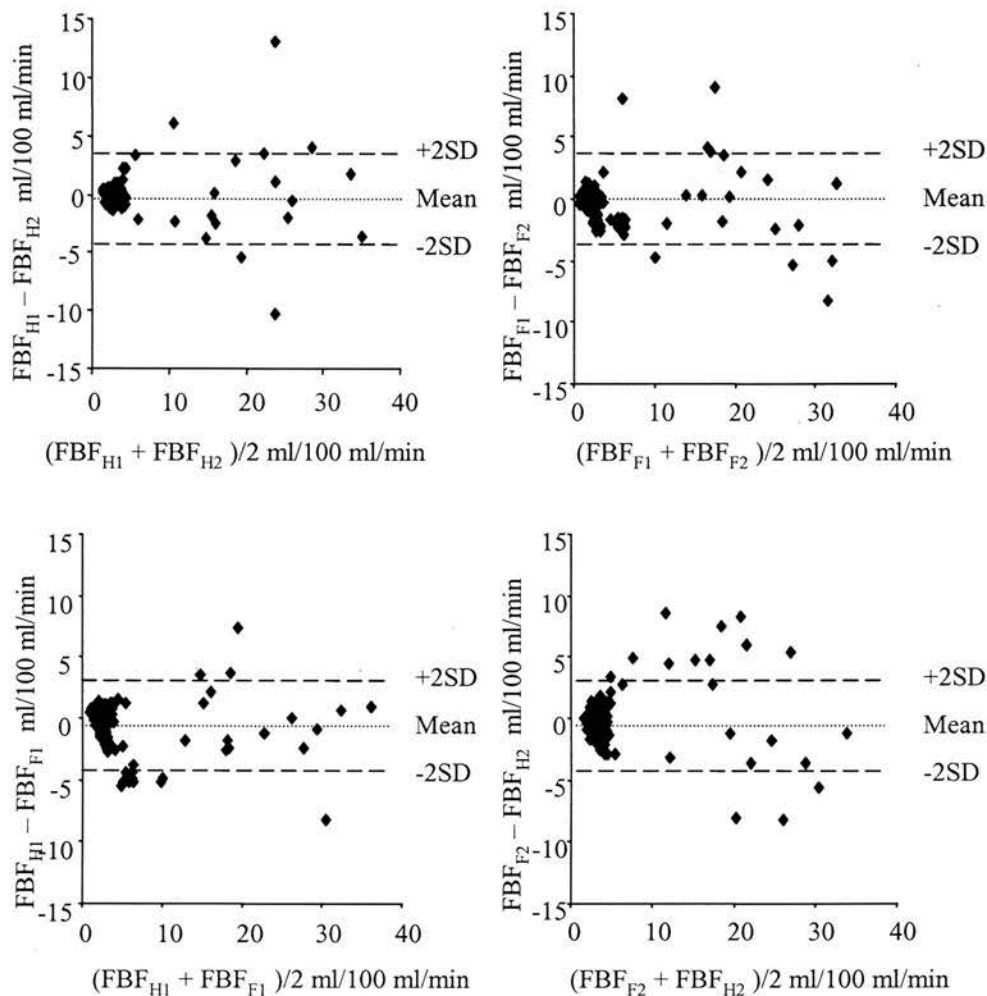


Figure 4.6

Bland-Altman comparison of (a) Hokanson EC4 (right arm) vs Hokanson EC4 (left arm) (b) Filtrass 2001 (right arm) vs Filtrass 2001 (left arm) (c) Filtrass 2001 (right arm) vs Hokanson EC4 (left arm) (d) Hokanson EC4 (right arm) vs Filtrass 2001 (left arm). Light dotted line indicates mean value with heavy dotted line indicates 95% confidence limits.

## 4.5 DISCUSSION

This study was designed to assess repeatability and systematic bias of a new device for measuring forearm blood flow by venous occlusion plethysmography. We have demonstrated that there was no significant difference in repeatability, as measured by difference in the changes in blood flow between left and right arms, when using the F2001 as compared to the HEC4. In addition, there was no systemic bias between the devices as assessed by Bland-Altman plots.

### 4.5.1 Forearm plethysmography

Forearm venous plethysmography has been used for nearly 100 years to assess forearm blood flow and is now a widely used and well validated technique (Joyner *et al* 2001). It has remained essentially unchanged over the last 50 years although advances have been made in certain areas to improve durability of equipment, ease of use and repeatability of results. The use of blood flow in the dominant forearm as a contemporaneous control for the effects on blood flow in the non-dominant arm and of intra-arterial drug infusion is widely used in the technique of forearm plethysmography [Benjamin *et al* 1995, Wilkinson & Webb 2001]. Indeed, it has been supported by this study with only ~ 1% difference in percentage change in FBF from baseline between left and right arms when measured by the HEC4. However, although the average difference between the arms was small, there was a spread of values which may reduce the power of studies to detect small absolute changes in FBF.

#### **4.5.2 Potential advantages of the F2001**

The F2001 is designed to measure forearm blood flow and among many potential improvements on previous devices. The F2001 device is more compact and portable than the HEC4. This may be of advantage in certain circumstances, such as in intensive care units or in studies where the equipment has to be moved between locations. Both types of strain gauge were easy to use, although the F2001 required greater manual dexterity to insert the monofilament into the body of the LVDT. The subject must remain still throughout the study because movement of the forearm can dislodge the strain gauges with either device. However, the HEC4 strain gauge may be simply replaced with little disruption to the study while disruption of the F2001 strain gauge requires re-calibration. This might be problematic if displacement occurred during a critical part of an interventional study. In terms of mechanical reliability, one F2001 strain gauge failed during the study (total 40 study days) while there were no failures of the HEC4 strain gauges. The upper cuff air inlet of the F2001 mechanically interfered with the strain gauge on inflation and the rate of arm and wrist cuff inflations were slower than the HEC4 (3-4 sec vs <1 sec). Slow inflating cuffs can result in venous engorgement if arterial occlusion is significantly delayed which can cause discomfort and could potentially affect results. However, neither of these problems appeared to occur in our study, suggesting the slower rate of cuff inflation is not of practical importance, at least with relatively low flows. Although there may be local variations, there was no significant cost difference between the two devices. In addition the F2001 does not use mercury in its construction and, therefore, has the advantage that its use would not be restricted in the future, were mercury in medical instruments to be banned [Padfield 1998].

## **Chapter 5**

### **Non-invasive measurement of cardiac output in patients with chronic heart failure**

**Leslie SJ, McKee S, Newby DE, Webb DJ, Denvir MA.** Non-invasive measurement of cardiac output in patients with chronic heart failure. *Blood Pressure Monitoring.* 2004;9(5):277-280.

## 5.1 SUMMARY

The measurement of cardiac output by thoracic bioimpedance has been assessed in several studies. However, there continues to be disagreement as to whether this technique is sufficiently accurate for use in clinical practice or research. The current study aimed to compare thoracic bioimpedance ( $CO_{TB}$ ) with thermodilution ( $CO_{TD}$ ) in 11 patients with stable chronic heart failure. Data are expressed as mean (SEM). A total of 282 measurements of cardiac output were analysed. Bland Altman analysis revealed an average difference between values of 0.3(2.2) L/min ( $p = 0.02$ ), suggesting a small average bias but marked variability in results. Similarly analysis of percentage change from baseline demonstrated a significant average difference between values of 10.1(30.1)%. There was no difference in between day repeatability between thermodilution and thoracic bioimpedance (-0.2(1.2) vs 0.1(1.0) L/min,  $p = 0.7$ ). This study demonstrates a correlation between the techniques but shows a poor level of agreement. The method of  $CO_{TB}$  underestimated cardiac output compared with  $CO_{TD}$ , and this difference appeared greater with higher cardiac outputs. Agreement was worse when results were expressed as change from baseline. This present study does not support the use of thoracic bioimpedance as an alternative to thermodilution in stable patients with chronic heart failure.

## 5.2 BACKGROUND

Measurement of cardiac output using the thermodilution technique is generally accepted as the 'gold standard' although it may not be suitable for use in certain situations, such as volunteer research studies, because of its invasive nature. In contrast, thoracic bioimpedance is a relatively simple, non-invasive technique for measuring cardiac output which has been employed in several clinical studies. However, there continues to be disagreement as to whether this technique is sufficiently accurate and precise to be of use in clinical practice or research.

Since the development of continuous cardiac output thermodilution catheters their use has become routine, not only in clinical practice but as a standard by which other less invasive techniques have been compared [Jacquet *et al* 1996, Lichtenthal *et al* 1996, Cariou *et al* 1998, Medin *et al* 1998, Neto *et al* 1999, Rocca *et al* 2001, Mets *et al* 2002, Bein *et al* 2004].

Many studies have supported the use of thoracic bioimpedance [Northridge *et al* 1990, Thomas *et al* 1992, Kööbi *et al* 1997, Salandin *et al* 1988, Belardinelli *et al* 1996, Barin *et al* 2000], while others suggest it is not sufficiently accurate [Doering *et al* 1995, Marik *et al* 1997, Spiering *et al* 1998]. The applicability of thoracic bioimpedance has been assessed in groups of unstable patients such as those following cardiac surgery [Kööbi *et al* 1997], in acute heart failure [Northridge 1990] and in critically ill patients on intensive care units [Kööbi *et al* 1997]. In addition, some workers have based assessment of clinical applicability on single point measurements rather than trends, [Salandin *et al* 1988, Marik *et al* 1997]. This may

limit their applicability as thoracic bioimpedance may be more accurate at measuring changes in cardiac output rather than absolute values [Thomas *et al* 1992].

There are few studies comparing thoracic bioimpedance with thermodilution for determining absolute, as well as relative changes in cardiac output in stable patients with chronic heart failure. The aim of this study was to compare the techniques of thoracic bioimpedance and thermodilution for the measurement of cardiac output in patients with stable chronic heart failure, during an acute study with a pharmacological intervention.

## **5.3 METHODS**

### **5.3.1 Patient selection**

Eleven patients with chronic heart failure (New York Heart Association Grade II/III) due to left ventricular dysfunction attended for a total of 30 visits (maximum 3 visits per patient). All patients had an ejection fraction  $\leq 35\%$  (by echocardiography using the biplanar Simpson's rule) and were stable on treatment for at least 3 months. All patients were in sinus rhythm and none had a cardiac pacemaker. The study was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. Written informed consent was obtained from each subject before entry into the study.

### **5.3.2 Measurements**

#### *5.3.2.1 Thermodilution*

Cardiac output was measured by the thermodilution technique using the semi-continuous single multi-lumen thermodilution cardiac output pulmonary artery catheter (Swan-Ganz CCOMbo - CCO/SVO<sub>2</sub>; Edwards Lifesciences LLC, Irvine, CA, USA) [Bottiger *et al* 1996, Ditmyer *et al* 1995]. This device has a thermal filament which transfers a safe level of heat to the surrounding blood in a pulsed, on-off manner every 30 s. Changes in the temperature of the blood distal to this are measured by a distal rapid-response thermistor in the pulmonary artery. The cardiac output is then calculated automatically using a modified Stewart-Hamilton equation [Yelderman *et al* 1993].



#### 5.3.2.2 *Thoracic bioimpedance*

Thoracic bioimpedance was performed using the BoMed<sup>®</sup> NCCOM3 system. (BoMed Medical Manufacturer Ltd., Irvine, CA, USA). Four pairs of low impedance electrodes are used, 2 on either side of the thorax at the level of the xiphoid sternum in the mid axillary line and 2 on the lateral aspect of the base of the neck. In each pair, the upper electrode delivers a small current which is sensed by the lower electrode, thus impedance is measured and the cardiac output can be calculated.

#### 5.3.3 **Protocol**

Patients were asked to attend after an overnight fast and to omit their regular medications on the morning of the study. The studies were conducted in a quiet room maintained at a constant temperature (22-24 °C). A pulmonary artery catheter was inserted, via a 9F femoral venous sheath, into the right pulmonary artery. Before starting the drug infusion, patients underwent an equilibration period of > 90 min until blood pressure, heart rate and cardiac output were stable, with 3 consecutive measurements within 10%. Systemic concentrations of endothelin antagonists were administered intravenously at low dose then high dose over 15 mins, 1 hour apart. Endothelin blockade causes vasodilatation and in patients with heart failure an increase in cardiac output (Chapter 10).

#### 5.3.4 **Data analysis and statistics**

Near simultaneous measurements of CO were obtained from the mean of 3 consecutive recordings at each period for both CO<sub>TD</sub> and CO<sub>TB</sub>. Data are expressed as mean value (standard error of the mean; SEM). Percentage changes in cardiac

output from baseline were also studied. Within device repeatability was assessed by analysing baseline measurements of cardiac output in the same patient on difference days. The method of Bland and Altman was used to describe the relationship between  $CO_{TD}$  and  $CO_{TB}$ . Bland-Altman analysis can be used to evaluate 2 techniques where the mean of the differences between 2 values is a measure of accuracy (or bias) and the standard deviation of these values gives a measure of precision [Bland 1996]. To avoid potential problems with linked data, individual measurements from patients at baseline and 75 mins were plotted. Statistical analysis was performed using paired, 2 tailed, Student's *t*-test. Statistical significance was taken at the 5% level.

## 5.4 RESULTS

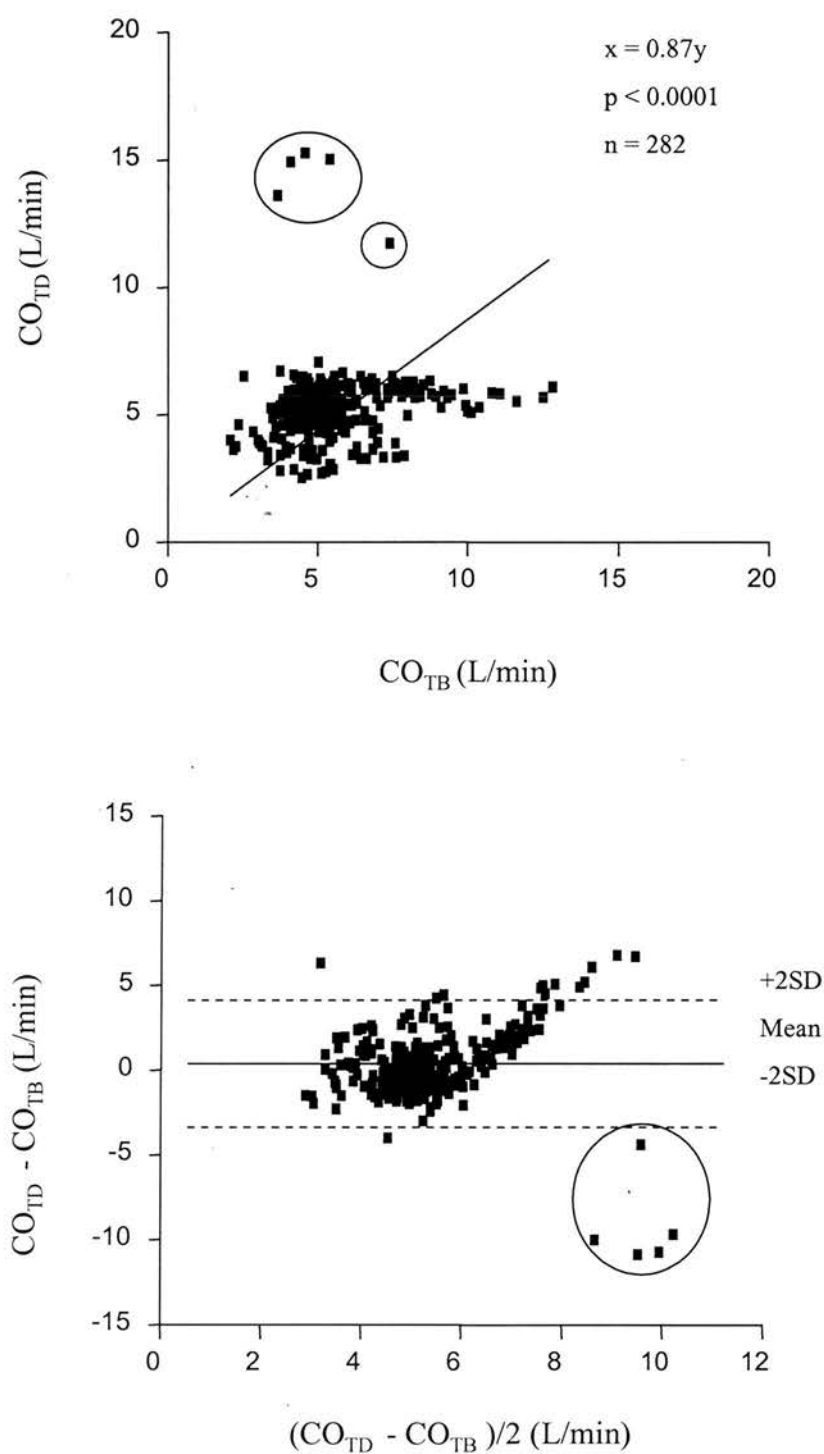
There were no adverse events and the study was well tolerated by all patients. Ten (91%) of the patients were male, and three (27%) had a NYHA score of II with the remaining 8 (73%) having a score of III. The other characteristics of the patients are presented in Table 1. The results of the haemodynamic effects of the study have been reported in Chapter 10. In brief, administration of systemic doses of endothelin antagonist resulted in peripheral vasodilation and an increase in cardiac output by a maximum of ~30%. The use of both selective and non-selective endothelin blockade at low and high dose allowed for a range of changes in cardiac output to be investigated.

### 5.4.1 Comparison between thoracic bioimpedance and thermodilution

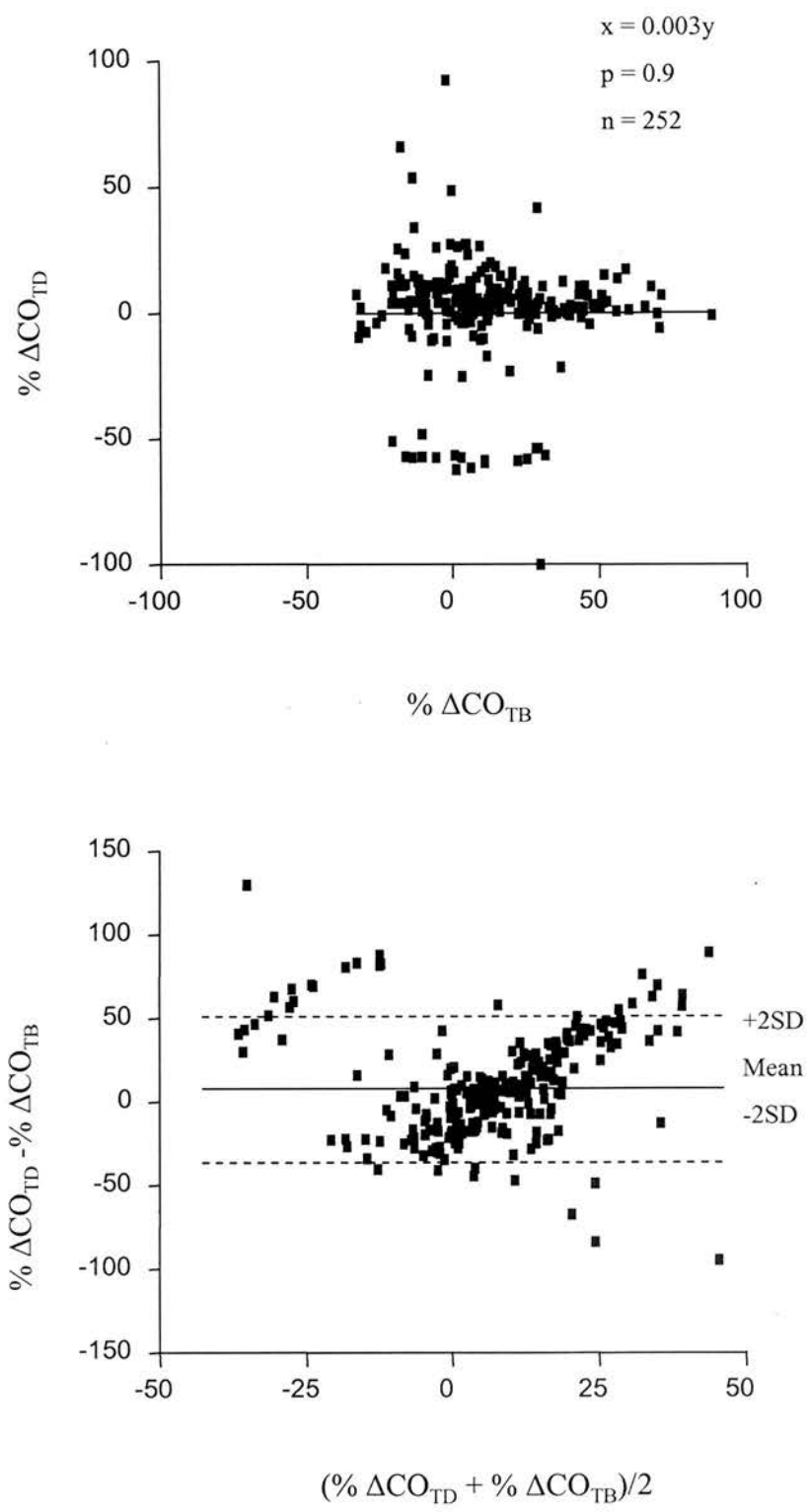
Correlation statistics demonstrated revealed reasonable correlation between the techniques ( $r=0.76$ ), however, Bland Altman analysis revealed an average difference between values of 0.3 (2.2) L/min ( $p = 0.02$ ), suggesting a small average bias but marked variability in results (Figure 5.1). Similarly analysis of percentage change from baseline demonstrated a significant average difference between values of 10.1 (30.1)% (Figure 5.2).

### 5.4.2 Repeatability of baseline measurements

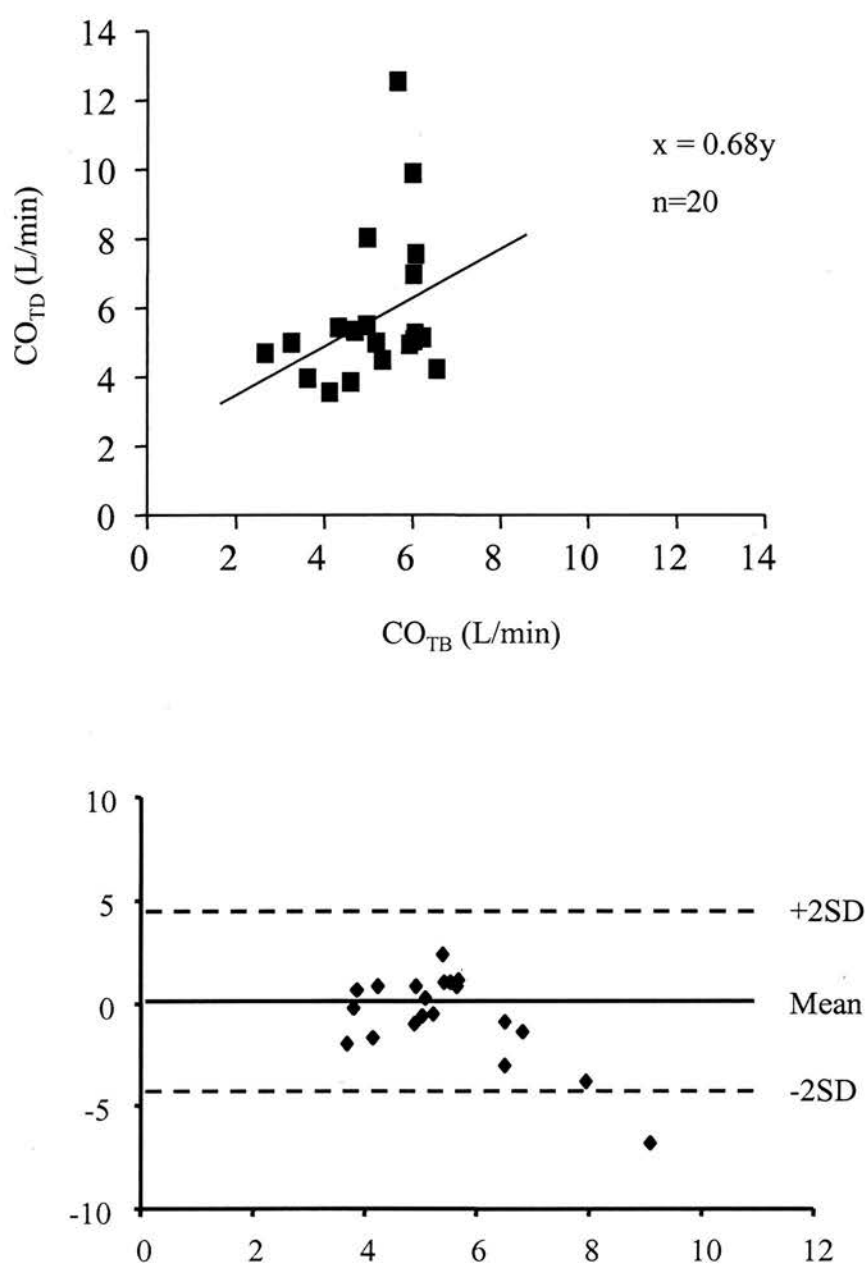
Within device repeatability was assessed by analysing baseline measurements of cardiac output in the same patient on difference days. There was no difference in between day repeatability between thermodilution and thoracic bioimpedance (-0.2 (1.2) vs 0.1 (1.0) L/min,  $p = 0.7$ ).



**Figure 5.1** Correlation and Bland Altman graphs of absolute cardiac output measurements. Potential outliers in circles



**Figure 5.2** Correlation and Bland Altman graphs of percentage change from baseline cardiac measurements



**Figure 5.3** Correlation and Bland Altman graphs of absolute cardiac output measurements at baseline and peak average cardiac output (t=75)

## 5.5 DISCUSSION

There is no 'perfect' method for measuring cardiac output although the technique of thermodilution is considered the 'gold standard'. However, due to the invasive nature of pulmonary artery catheterisation there is considerable interest in the non-invasive technique of thoracic bioimpedance. Thoracic bioimpedance has the advantage that it is relatively inexpensive and easy to use, however, its accuracy has been questioned.

This study has compared the techniques of thoracic bioimpedance against thermodilution in stable patients with chronic heart failure undergoing an acute haemodynamic intervention study. A meta-analysis of thoracic bioimpedance studies found a good correlation between thoracic bioimpedance and reference techniques ( $r = 0.66$ ) [Fuller *et al* 1992]. In keeping with these studies we have found a good correlation between thoracic bioimpedance and a thermodilution technique ( $r = 0.76$ ). However, while there is a positive correlation between the techniques, there is a poor level of agreement. The method of thoracic bioimpedance systematically underestimated cardiac output compared with thermodilution, and this difference appeared to be greater with higher cardiac output. Several studies have supported the use of thoracic bioimpedance when measuring trends in cardiac output [Northridge *et al* 1990, Thomas *et al* 1992, Kööbi *et al* 1997, Salandin *et al* 1988, Belardinelli *et al* 1996, Barin *et al* 2000]. However, the current study does not support this finding with poor agreement between values when expressed as percentage change from baseline despite reasonable repeatability of baseline measurements.

We observed an unacceptably low level of agreement both for absolute and changes in cardiac output. Previous studies have assessed thoracic bioimpedance in critically ill patients or patients after mechanical ventilation or undergoing cardiac surgery. Pulmonary oedema and mechanical ventilation can affect thoracic bioimpedance and many previously studied patients may have been unsuitable for measurement of cardiac output by thoracic bioimpedance. The patients in this present study had stable chronic heart failure, were in sinus rhythm and in the controlled setting of an acute haemodynamic intervention study. It would have been expected that these optimal conditions would provide the best opportunity for thoracic bioimpedance to be comparable with the gold standard of thermodilution, given that patients were stable, self-ventilating and had no pulmonary oedema. However, the findings suggest that thoracic bioimpedance is not comparable to thermodilution in patients with stable chronic heart failure. In addition, invasive monitoring using pulmonary artery catheters may have additional benefits over non-invasive thermodilution in that they allow direct measurement of blood pressure and allow sampling of blood from the pulmonary artery.

One of the most important confounding features in heart failure patients when measuring cardiac output by thoracic bioimpedance is changes in fluid status and the presence of pulmonary oedema. It would have been of interest to compare thoracic fluid volume against pulmonary capillary wedge pressure, but these data were not collected during the study.



Some of the differences noted in our study may be due to slight difference in the timing of cardiac output measurements, as thoracic bioimpedance cannot be measured at exactly the same time as thermodilution. However, measurements were made within 60s of each other and such a small difference in time is unlikely to explain the degree of poor agreement between these two techniques.

In conclusion, thoracic bioimpedance using the BoMed system, does not appear to sufficiently agree with invasive thermodilution techniques for measuring cardiac output in patients with stable chronic heart failure. Therefore, it should not be routinely used as an alternative to thermodilution in patients with heart failure even in the controlled setting of a clinical study. However, there continues to be improvements in the technology associated with the technique of thoracic bioimpedance, which, combined with its non-invasive nature may make it suitable for use in the future.

## Chapter 6

### **Endothelins and their inhibition in the human skin microcirculation: ET<sub>[1-31]</sub> a new vasoconstrictor peptide**

**Leslie SJ**, Rahman M, Denvir MA, Newby DE, Webb DJ. Endothelins and their inhibition in the human skin microcirculation. *Br J Clin Pharmacol* 2004;57:720-725.

## 6.1 SUMMARY

Endothelin-1 (ET-1<sub>[1-21]</sub>) is an extremely potent vasoconstrictor in the human skin microcirculation and is generated from larger precursor peptides. The aims of the present study were to assess the vasoactive effects of these precursors as well as endothelin blockade in the human skin microcirculation, *in vivo*. Six healthy volunteers received intradermal injections of a range of doses of big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub>, ET-1<sub>[1-21]</sub>, BQ-123 (ET<sub>A</sub> receptor antagonist), BQ-788 (ET<sub>B</sub> receptor antagonist), phosphoramidon (endothelin converting enzyme (ECE) inhibitor) or saline control (0.9 %). Skin blood flow (SBF) was measured using standard laser Doppler flowmetry. Big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub> and ET-1<sub>[1-21]</sub> reduced SBF when compared with saline control ( $p < 0.01$  for all). Big ET-1<sub>[1-38]</sub> was ~30 fold, and ET-1<sub>[1-31]</sub> ~150 fold less potent than ET-1<sub>[1-21]</sub> as defined by skin vasoconstriction. Phosphoramidon, BQ-123 and BQ-788, given alone, all caused vasodilatation in the human skin microcirculation ( $p < 0.01$  for all). In the human skin microcirculation, big ET-1<sub>[1-38]</sub> and ET-1<sub>[1-31]</sub> are less potent vasoconstrictors than ET-1<sub>[1-21]</sub>. The effects of big ET-1<sub>[1-38]</sub> and phosphoramidon suggest the presence of endogenous ECE activity in the skin. In contrast to skeletal muscle resistance vessels, ET-1<sub>[1-21]</sub> contributes to the maintenance of skin microvascular tone through both ET<sub>A</sub> and ET<sub>B</sub> receptor mediated vasoconstriction.

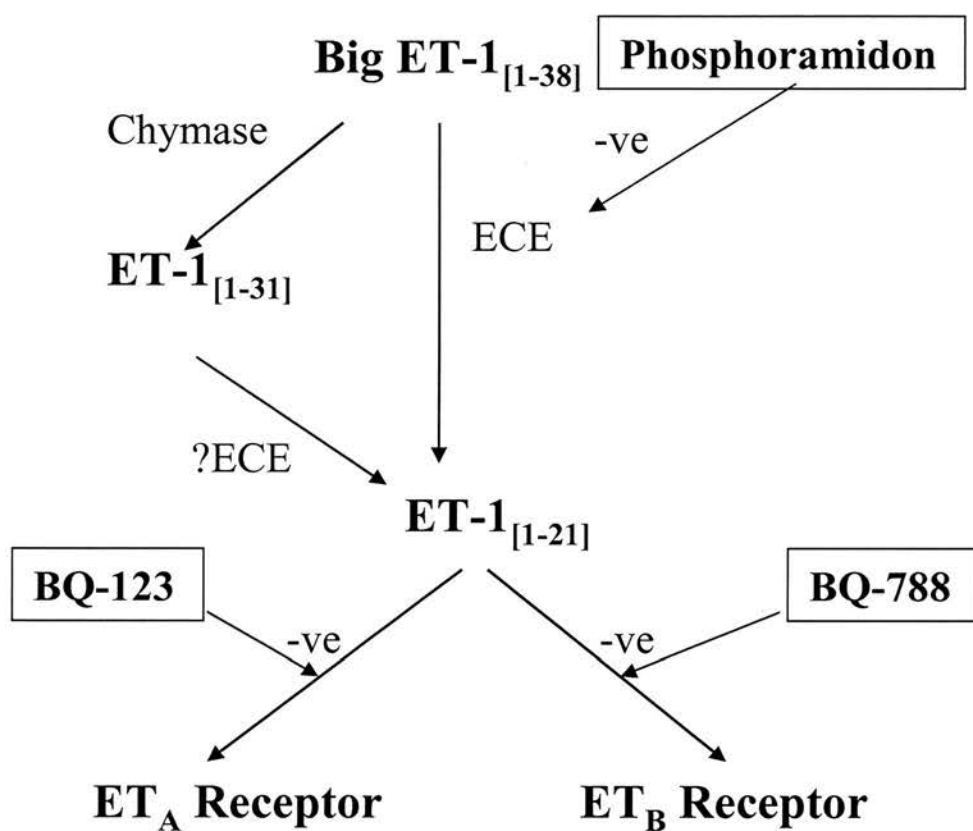
## 6.2 BACKGROUND

Endothelin-1<sub>[1-21]</sub> (ET-1) is a potent vasoconstrictor in several vascular beds including the skin microcirculation [Wenzel 1994 & 1996]. It is formed by the action of endothelin converting enzyme (ECE) on its inactive precursor ET-1<sub>[1-38]</sub> (big ET-1) (Figure 6.1). There are 2 distinct endothelin receptors subtypes; ET<sub>A</sub> and ET<sub>B</sub>. Both are present on vascular smooth muscle cells mediating vasoconstriction while ET<sub>B</sub> is also present on endothelial cells mediating vasodilatation. Thus, the cardiovascular effects of ET-1<sub>[1-21]</sub> will depend, in part, on the balance of action at these 2 receptors and in particular the balance between the vasodilating and vasoconstricting actions of the ET<sub>B</sub> receptor.

The endothelin system may be involved in the pathophysiology of several cardiovascular diseases including hypertension [Haynes *et al* 1994], renal failure [Hand *et al* 1999], pulmonary hypertension [McCulloch *et al* 1998] and chronic heart failure [Wei *et al* 1994]. Blockade of the system remains an area of major interest. In particular, whether selective or dual receptor antagonism or ECE inhibition will provide the best treatment strategy remains unclear. Initial efforts have been focused mainly on endothelin receptor blockade. Endothelin receptor antagonists have benefits in patients with primary pulmonary hypertension [Channick *et al* 2001] although results in patients with chronic heart failure have so far been disappointing [Coletta *et al* 2002]. Clinical development of ECE inhibitors continues but there are, as yet, no fully published data on their clinical efficacy.

Recently, ET-1<sub>[1-31]</sub>, a new derivative of big ET-1, has been identified in humans. It is generated following the cleavage of big ET-1 at the Tyr<sub>31</sub> - Gly<sub>32</sub> bond by human chymase [Nakano *et al* 1997] and may represent an active intermediary in an alternative pathway of ET-1 production (Figure 1). In cultured human coronary artery smooth muscle cells [Yoshizumi *et al* 1998, Inui *et al* 1999] and human mesangial cells [Yasuoka *et al* 1999], ET-1<sub>[1-31]</sub> increases intracellular calcium. ET-1<sub>[1-31]</sub> causes vasoconstriction in isolated porcine coronary arteries [Kishi *et al* 1998], monkey trachea [Takai *et al* 1998], human umbilical arteries [Takeji *et al* 2000] as well as human coronary and mammary arteries [Maguire *et al* 2001]. Data suggest that ET-1<sub>[1-31]</sub> may be cleaved to ET-1<sub>[1-21]</sub> for its biological activity in cultured bronchial smooth muscle cells [Hayasaki-Kajiwara *et al* 1999] and in both guinea-pig [Honore *et al* 2002] and human arteries [Maguire *et al* 2001]. However, to date there have been no *in vivo* clinical studies to assess the cardiovascular effects of ET-1<sub>[1-31]</sub>.

The aims of this study were to investigate the *in vivo* vascular effects of ET-1<sub>[1-21]</sub>, its precursors big ET-1<sub>[1-38]</sub> and ET-1<sub>[1-31]</sub>, and blockade of endogenous ET-1 activity by BQ-123 (a selective ET<sub>A</sub> receptor antagonist) [Ihara *et al* 1992], BQ-788 (a selective ET<sub>B</sub> receptor antagonist) [Ishikawa *et al* 1994] and ET-1 generation by phosphoramidon (an ECE inhibitor) in the human skin microcirculation.



**Figure 6.1** Pathway of production of ET-1<sub>[1-31]</sub>

## **6.3 METHODS**

### **6.3.1 Subjects**

Six healthy men (age range 20 - 30 years), with no risk factors for vascular disease, participated in each study. Written informed consent was obtained and studies were performed with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. None of the subjects were taking regular medication and all avoided medication for 1 week before each study. All subjects abstained from alcohol for 24 hours and from food, caffeine and tobacco for at least 12 hours before each study.

### **6.3.2 Skin blood flow measurement**

Skin blood flow was assessed using standard laser Doppler skin flowmetry (2 channel, MBF 3D, Moor Instruments Ltd, UK) at baseline and every 2 min for the first 10 min and then every 5 min up to 60 min. Voltage output from the Doppler flowmeter was calibrated with standard flux solution (Moor Instruments Ltd, UK) and transferred to a Macintosh personal computer (Classic II, Apple Computer Inc, USA) with a MacLab analogue-to digital converter and 'CHART' software (v.3.28, AD Instruments, New Zealand). Signals were averaged over 20 seconds at each time point.

### **6.3.3 Study drugs**

ET-1<sub>[1-31]</sub> (Peptide Institute, Osaka, Japan), and big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-21]</sub>, BQ-123, BQ-788 and phosphoramidon (Clinalfa, Laufelfingen, Switzerland) were dissolved in physiological saline (0.9%; Baxter Healthcare Ltd., Thetford, UK), which was also

used as the vehicle control. Phosphoramidon was poorly soluble allowing a limited dose-range to be examined.

#### **6.3.4 Study protocol**

Subjects rested recumbent in a quiet room maintained at a constant temperature of 22 - 24 °C for 15 min to allow stabilisation of skin blood flow. Four sites for injection were identified and marked on the volar aspect of each forearm. Care was taken to avoid underlying veins (demonstrated by high baseline Doppler signals) and arteries (demonstrated by pulsatile Doppler signals). A laser probe holder was attached to each of the 4 sites on the forearm the skin using adhesive tape designed to reduce probe movement during the study. Baseline blood flow was measured. A measure of 'biological zero' was obtained by arterial occlusion by inflating an upper arm cuff to supra-systolic pressures. Sufficient time ~15 min was allowed after arterial occlusion to allow a return to baseline blood flow. All study drugs were administered by 10 µL intradermal injection (0.33 mm [29.5 SWG] needle; Becton-Dickinson, Dublin, Ireland). Following dose-ranging pilot studies, subjects received, in random order, either saline control or study drug over a range of concentrations; big ET-1<sub>[1-38]</sub> (0.1 to 30 pmol), ET-1<sub>[1-31]</sub> (1 pmol to 0.3 nmol), ET-1<sub>[1-21]</sub> (1 amol to 1 pmol), BQ-123 (0.1 to 30 nmol), BQ-788 (0.1 to 30 nmol) and phosphoramidon (0.1 to 10 nmol). The maximum dose of phosphoramidon was limited by solubility.

#### **6.3.5 Data handling and statistical analysis**

Results are expressed in arbitrary perfusion units (PU). Intradermal injection of saline placebo causes an increase in laser Doppler signal [Wenzel *et al* 1994] and



therefore all results are presented as placebo corrected mean  $\pm$  SEM. Area under the curve (AUC) for the response between 0 and 30 min was used to determine potency. Potency was estimated as the dose required to cause a significant vasoconstriction in the skin compared with saline placebo. Statistical difference was tested by ANOVA with repeated measures over time and paired, 2 tailed, Student's *t*-test with correction for repeated measures (Excel 5.0, Microsoft Ltd, USA). A *p* value < 0.05 was considered statistically significant.

## 6.4 RESULTS

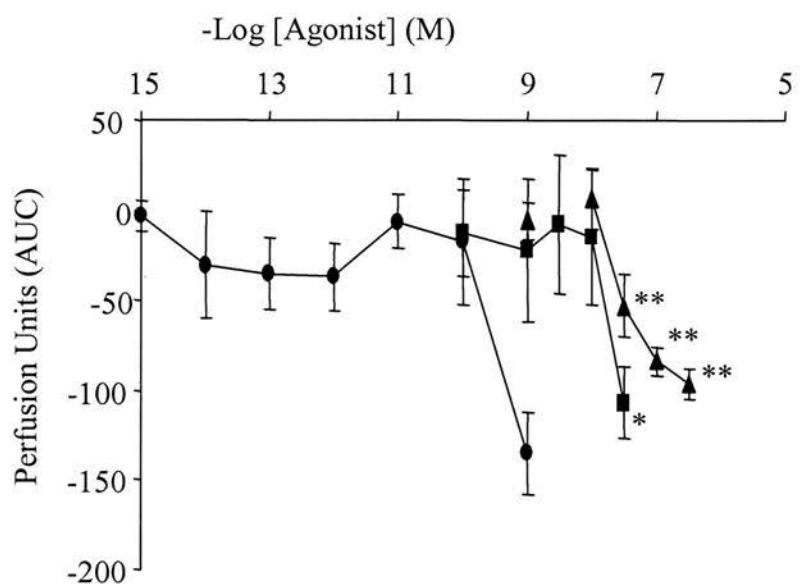
Intradermal drug delivery was well tolerated by all volunteers. Transient discomfort occurred at some injection sites but was unrelated to injectate and did not persist for longer than 10 seconds. This discomfort was not associated with impaired tissue viability and did not appear to affect responses.

### 6.4.1 Effect of endothelin agonists

Big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub> and ET-1<sub>[1-21]</sub> caused vasoconstriction that was visually evident on the skin, causing a marked area of pallor. Big ET-1<sub>[1-38]</sub> was ~ 30 fold and ET-1<sub>[1-31]</sub> was ~150 fold less potent than ET-1<sub>[1-21]</sub> (Figure 6.2a). Compared with control, sustained reduction in blood flow was caused by big ET-1<sub>[1-38]</sub> (30 pmol; maximum decrease  $25 \pm 8$  PU,  $p = 0.04$ ), ET-1<sub>[1-31]</sub> (0.3 nmol; maximum decrease  $13 \pm 3$  PU,  $p = 0.04$ ) and ET-1<sub>[1-21]</sub> (1 pmol; maximum decrease  $17 \pm 4$  PU,  $p = 0.003$ ) (Figure 6.2b). At these concentrations, vasoconstriction was sustained and was still visibly present at 24 hrs, although the duration of response beyond 60 min was not formally assessed.

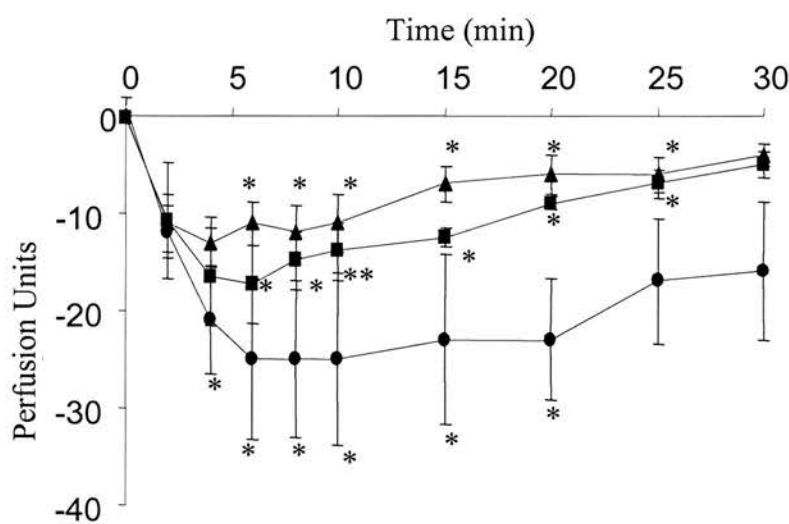
### 6.4.2 Effect of endothelin blockade

BQ-123 and BQ-788 caused vasodilatation and appeared to be equipotent (Figure 6.3a). Compared with control, a sustained increase in blood flow was caused by BQ-123 (300 nmol; maximum increase  $30 \pm 5$  PU,  $p = 0.002$ ) and BQ-788 (300 nmol; maximum increase  $18 \pm 5$  PU,  $p = 0.004$ ) (Figure 6.3b). Compared with control, phosphoramidon caused a small increase in blood flow at the highest dose (10 pmol; maximum increase  $11 \pm 2$  PU,  $p = 0.009$ , Figure 6.3a,b).



a)

● ET-1<sub>[1-21]</sub>  
 ▲ ET-1<sub>[1-31]</sub>  
 ■ Big ET-1<sub>[1-38]</sub>

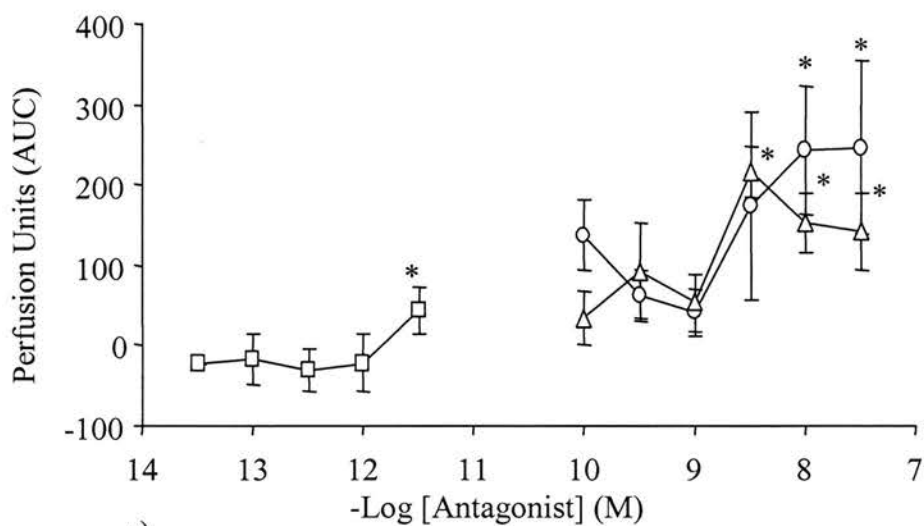


b)

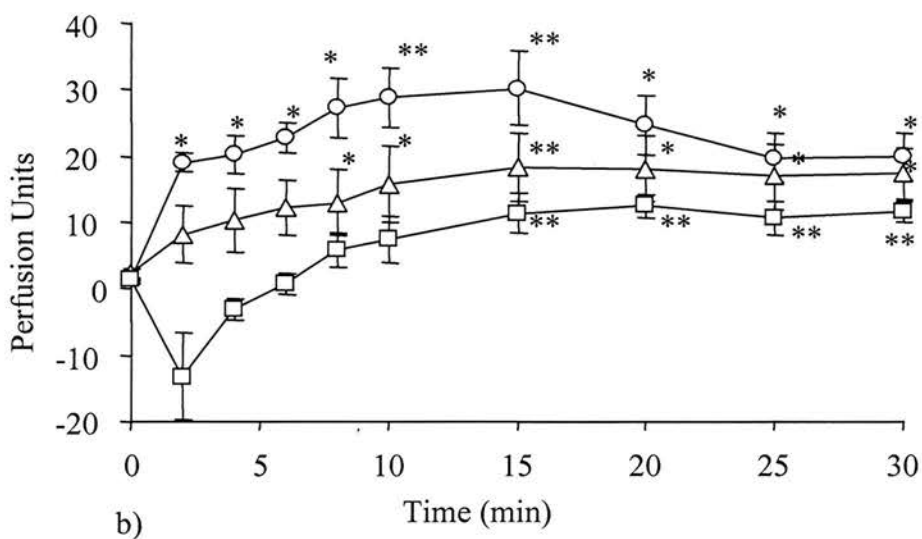
**Figure 6.2** Effects of ET agonists on skin blood flow.

a) dose ranging study, \* =  $p < 0.05$ , \*\*  $p < 0.01$ . (AUC, ANOVA)

b) time course of maximum dose \* =  $p < 0.05$ , \*\*  $p < 0.01$  two tailed paired Student's  $t$ -test with correction for repeated measured



—○— BQ-123  
 —△— BQ-788  
 —□— Phosphoramidon



**Figure 6.3** Effects of ET antagonists on skin blood flow.

a) dose ranging study, \* =  $p < 0.05$ , \*\*  $p < 0.01$ . (AUC, ANOVA)

b) time course of maximum dose \* =  $p < 0.05$ , \*\*  $p < 0.01$  two tailed paired Student's *t*-test with correction for repeated measured

## 6.5 DISCUSSION

In the human skin microcirculation, we have confirmed that ET-1<sub>[1-21]</sub> is a potent vasoconstrictor and shown for the first time that ET-1<sub>[1-31]</sub> and big ET-1<sub>[1-38]</sub> also cause skin vasoconstriction *in vivo*. Our results suggest that there is ECE activity in the skin as demonstrated by the vasoconstriction following intradermal administration of big ET-1<sub>[1-38]</sub> and vasodilatation with ECE inhibition. In addition, we have confirmed that selective blockade of the ET<sub>A</sub> receptor caused skin microvasculatory vasodilation [Wenzel *et al* 1998a] and shown directly that selective blockade of the ET<sub>B</sub> receptor also results in vasodilatation. In contrast to observations in resistance vessels, this suggests that ET<sub>B</sub> receptors in the skin contribute to ET-1 mediated vasoconstriction not only in arterial disease [Wenzel *et al* 1998a] but also in healthy blood vessels.

The novel finding that ET-1<sub>[1-31]</sub> is a vasoconstrictor is of interest, and the first evidence of its vasoactive properties *in vivo* in man. If ET-1<sub>[1-31]</sub> is converted to ET-1<sub>[1-21]</sub> by a non-ECE pathway and if this contributes importantly to ET-1 generation then specific receptor blockade may offer greater functional activity than ECE inhibition. Alternatively, ET-1<sub>[1-31]</sub> may have vasoconstricting activity of its own at endothelin receptors. Recently, the vasoconstricting effects of ET-1<sub>[1-31]</sub> have been shown to be mediated via the ET<sub>A</sub> receptor in rabbit renal resistance vessels [Ozawa *et al* 2003], an effect which was unaffected by phosphoramidon. Although ET-1<sub>[1-31]</sub> is less potent than ET-1<sub>[1-21]</sub>, as a vasoconstrictor, local production of ET-1<sub>[1-31]</sub> may occur specifically in tissues that express human chymase, such as from the mast cells within the shoulder region of coronary atheromatous plaques [Karttinen *et al* 1994,

Kovanen *et al* 1995]. Therefore, generation of ET-1<sub>[1-31]</sub> could contribute to coronary artery spasm at the time of plaque rupture. Indeed, ET-1<sub>[1-31]</sub> may be cleaved to ET-1<sub>[1-21]</sub> for its biological activity in cultured bronchial smooth muscle cells [Hayasaki-Kajiwara *et al* 1999] and in both guinea-pig [Honore *et al* 2002] and human arteries [Maguire *et al* 2001].

Given that big ET-1<sub>[1-38]</sub> has affinity for the endothelin A receptor which is 1000 fold less than ET-1<sub>[1-21]</sub> [Ishikawa *et al* 1994], that big ET-1<sub>[1-38]</sub> was ~30 fold less potent than ET-1<sub>[1-21]</sub> makes it unlikely that big ET-1<sub>[1-38]</sub> caused a major direct vasoconstrictor action and suggests that there is some conversion of big ET-1 to ET-1<sub>[1-21]</sub> in the skin. It has previously been demonstrated that big ET-1<sub>[1-38]</sub> is vasoactive in human arteries [Haynes *et al* 1994] but not veins [Haynes *et al* 1995] and that this vasoconstriction can be blocked by ECE inhibition [Haynes *et al* 1994]. The presence of ECE activity in the skin is further supported in this present study by the vasodilatory effects of phosphoramidon. This is also consistent with preliminary clinical studies where systemic ECE inhibition caused systemic vasodilatation and a reduction in blood pressure [de Voogd *et al* 2001]. In our study there appeared to be a transient vasoconstriction to phosphoramidon although this was not consistent and did not reach statistical significance. This observation may warrant further investigation as the current study was underpowered to investigate such a small transient change.

Endothelin antagonists have been studied in the skin by other groups. The selective ET<sub>A</sub> receptor antagonist PD147953 and the non-selective endothelin receptor

antagonist PD145065 caused vasodilatation and attenuated the vasoconstrictor effects of ET-1<sub>[1-21]</sub> in the human skin *in vivo* [Wenzel *et al* 1994 & 2001]. BQ-123 is a selective ET<sub>A</sub> receptor antagonist and has been shown in many vascular beds [Berrazueta *et al* 1997], to be a vasodilator by blocking the effects of ET-1<sub>[1-21]</sub>. The present study demonstrates that BQ-123 causes vasodilatation, confirming that ET-1 contributes to the maintenance of basal vascular tone in the skin of healthy volunteers. BQ-788 also caused skin vasodilatation. This result was unexpected as selective ET<sub>B</sub> receptor antagonism causes vasoconstriction in skeletal muscle [Verhaar *et al* 1998] and when given systemically [Strachan *et al* 1999]. In addition, previous data in the skin microcirculation have demonstrated that ET-1 mediates vasoconstriction via the ET<sub>A</sub> receptor with little contribution from the ET<sub>B</sub> receptor [Wenzel *et al* 2001, Lipa *et al* 1999]. However, our data indicate that the dominant effect of ET-1<sub>[1-21]</sub> on the ET<sub>B</sub> receptor is vasoconstriction in the human skin microcirculation, and that blockade of the ET<sub>B</sub> receptor results in vasodilatation. It is possible that some of this effect may be a result of displacement of ET-1 from ET<sub>B</sub> receptors and a reduction of available binding sites, thereby increasing vasoconstriction via the ET<sub>A</sub> receptor. This finding may require further investigation.

Endothelin receptor antagonists have proven benefits in patients with primary pulmonary hypertension [Channick *et al* 2001] and reduce blood pressure in hypertensive patients [Krum *et al* 1998]. To date, clinical trials investigating the potential benefits of endothelin receptor antagonists in CHF have been disappointing [Coletta *et al* 2002]. ECE inhibitors inhibit endothelin production and provide a potential alternative to endothelin receptor antagonism. Unlike receptor blockade

which can increase plasma ET-1<sub>[1-21]</sub> concentrations, ECE inhibitors have the advantage that they block ET-1<sub>[1-21]</sub> production, thus reducing plasma ET-1<sub>[1-21]</sub> concentrations and leaving clearance receptors unblocked. However, the effects of raised plasma concentration of ET-1<sub>[1-21]</sub>, associated with ET<sub>B</sub> receptor blockade [Verhaar *et al* 1998], may not be relevant if ET<sub>A</sub> receptors are also blocked. However, if there are clinically significant non-ECE pathways of production with vasoactive intermediary peptides such as ET-1<sub>[1-31]</sub> then ECE inhibition may be a less attractive treatment strategy.

While the doses of peptides used in these experiments are known, the concentration when injected into the skin cannot be measured, due to the unknown volume of distribution in the skin. However, as the same volume of injectate was used for each dose, comparing the relative potencies of each peptide is probably justified. Furthermore, true potency may vary depending on whether compounds are full or partial agonists. Highly selective antagonists of each receptor were not available to us. Although BQ-788 might have blocked ET<sub>A</sub> receptor function, its 100 fold greater affinity for the ET<sub>B</sub> receptor [Russell *et al* 1996] compared with the ET<sub>A</sub> receptor, and the equal potency of BQ-123 and BQ-788 in this study suggests that BQ-788 does indeed mediate vasodilatory effects via blockade of the ET<sub>B</sub> receptor. Another limitation is that phosphoramidon is not only an inhibitor of ECE but also an inhibitor of neutral endopeptidase (NEP). However, whether in health or cardiovascular disease [Haynes *et al* 1994, Kentsch *et al* 1996, Spratt *et al* 2001] the inhibition of NEP tends to cause vasoconstriction and therefore if anything led to an underestimate of the vasodilator effects of selective ECE inhibition.



In summary, the discovery of a vasoactive intermediary endothelin peptide may have importance for the further development of endothelin blockers as clinical therapies. The skin microcirculation provides an opportunity to investigate the vasoactive properties of compounds, *in vivo*, in a relatively safe manner due to the small doses which are administered. However, further studies are required to determine whether ET-1<sub>[1-31]</sub> is vasoactive in other vascular beds. In particular it will be of interest to note whether ET-1<sub>[1-31]</sub> causes vasoconstriction in resistance blood vessels.

## Chapter 7

### **Endothelin-1<sub>[1-31]</sub> is not elevated in men with chronic heart failure**

**Leslie SJ**, Johnston N, Strachan FE, Gray GA, Newby DE, Denvir MA, Webb DJ. Endothelin-1<sub>[1-31]</sub> is not elevated in men with chronic heart failure. *J Cardiovasc Pharmacol* 2004;44:S96-99.

## 7.1 SUMMARY

Endothelin-1<sub>[1-31]</sub> (ET-1<sub>[1-31]</sub>) is a recently discovered member of the endothelin family with vasoactive properties in several animal models and *in vivo* in man. It is generated from big endothelin-1 (big ET-1<sub>[1-38]</sub>) by human mast cell chymase and may be a novel intermediary peptide in the production of ET-1<sub>[1-21]</sub>. Given that both big ET-1<sub>[1-38]</sub> and chymase activity are increased in chronic heart failure (CHF), the aim of this study was to determine whether plasma ET-1<sub>[1-31]</sub> concentrations are elevated in patients with CHF. Plasma ET-1<sub>[1-31]</sub> concentrations were measured by enzyme linked immunosorbent assay in 9 patients with CHF, and 9 age and sex matched control subjects. Consistent with previous studies, plasma concentrations of big ET-1<sub>[1-38]</sub> were elevated in patients compared with controls ( $17.1 \pm 4.4$  vs  $8.9 \pm 3.4$  pg/ml,  $p = 0.002$ ), although there were no differences in plasma ET-1<sub>[1-21]</sub> ( $3.3 \pm 0.4$  vs  $3.4 \pm 0.7$  pg/ml,  $p = 0.7$ ) or ET-1<sub>[1-31]</sub> (both  $1.1 \pm 0.1$  pg/ml,  $p = 0.2$ ) concentrations. We have demonstrated that patients with CHF have normal plasma ET-1<sub>[1-31]</sub> concentrations. This suggests that, in contrast to big ET-1<sub>[1-38]</sub>, plasma ET-1<sub>[1-31]</sub> is unlikely to be a useful prognostic marker in patients with CHF.

## 7.2 BACKGROUND

New derivatives of endothelin have recently been characterised in humans. In contrast, to the generation of endothelin -1 (ET-1<sub>[1-21]</sub>) by cleavage of the inactive precursor big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub> is generated following the cleavage of big ET-1<sub>[1-38]</sub> at the Tyr<sup>31</sup>-Gly<sup>32</sup> bond by human chymase [Nakano *et al* 1997]. This endothelin intermediary peptide may be of clinical importance in conditions such as chronic heart failure where there is increased generation of big ET-1<sub>[1-38]</sub> and increased chymase activity in tissues including the heart [Urata *et al* 1990] and blood vessels [Urata *et al* 1995]. In addition, there are increased numbers of mast cells and human mast cell chymase at the shoulder regions of unstable coronary artery plaques [Kovanen *et al* 1995, Kaartinen *et al* 1994] and ET-1<sub>[1-31]</sub> may also contribute to the pathogenesis of ischaemic heart disease and acute coronary syndromes.

*In vitro* studies have shown that ET-1<sub>[1-31]</sub> is a vasoconstrictor in isolated porcine coronary arteries [Kishi *et al* 1998], rabbit pulmonary arteries [Hanson *et al* 1996] and monkey trachea [Takai *et al* 1998]. In cultured human coronary artery smooth muscle cells [Yoshizumi *et al* 1998, Inui *et al* 1999] and human mesangial cells [Yasuoka *et al* 1999], ET-1<sub>[1-31]</sub> increases intracellular calcium. In addition, ET-1<sub>[1-31]</sub> causes vasoconstriction in human umbilical arteries [Takeji *et al* 2000] and we have previously reported that ET-1<sub>[1-31]</sub> is a vasoconstrictor in the human skin microcirculation *in vivo* [Chapter 6]. It is likely that ET-1<sub>[1-31]</sub> causes vasoconstriction via endothelin A, and perhaps the endothelin B, receptors either directly or following conversion to ET-1<sub>[1-21]</sub>.

Recently, a new sensitive and selective enzyme linked immunosorbent assay (ELISA) has been developed to detect ET-1<sub>[1-31]</sub> [Okishima *et al* 1999]. The aims of

this study were, therefore, to validate the measurement of plasma ET-1<sub>[1-31]</sub> using this ELISA, and to quantify circulating plasma ET-1<sub>[1-31]</sub> concentrations in patients with chronic heart failure and healthy matched controls.

## **7.3 METHODS**

### **7.3.1 Subjects**

Nine male patients with moderate to severe chronic left ventricular dysfunction who had been stable on therapy for at least 3 months were recruited. They had a left ventricular ejection fraction of  $< 35\%$ , shortening fraction of  $< 20\%$  or left ventricular end diastolic diameter  $> 5.6$  cm. Patients were compared with age and sex matched healthy controls. The study was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. Written informed consent was obtained from each subject before entry into the study.

### **7.3.2 Blood sampling and plasma extraction**

All subjects omitted their medications on the day of study and attended after an overnight fast. Venous blood samples were collected into ethylene diamine tetraacetic acid (EDTA; Sarstedt, Aktiengesell Schaft & Co, Germany), placed on ice and plasma immediately separated by centrifugation (2500 *g* for 20 min at 4°C). Plasma samples were stored at -80 °C until analysis. Plasma was acidified with an equal volume of 20% acetic acid before being extracted using methanol activated Bond Elut C18 columns (Varian, Harbor City, CA, USA). The columns were perfused with deionised water and 10% acetic acid prior to the addition of plasma samples. The columns were then washed with 10% acetic acid and ethyl acetate, and the bound endothelin was eluted with 80% methanol: 20% ammonium bicarbonate. The eluate was dried down under a continuous stream of nitrogen at 37°C and the dried eluates reconstituted with assay buffer. Recovery fraction was estimated by

adding known quantities of ET-1<sub>[1-31]</sub> to plasma. The assays were performed with the kind assistance of Mr Neil Johnston.

### 7.3.3 Peptides

ET-1<sub>[1-31]</sub> (Peptide Institute Inc., Osaka, Japan), big ET-1<sub>[1-38]</sub> (Clinalfa, Läufelfingen, Switzerland) and ET-1<sub>[1-21]</sub> (Clinalfa) were dissolved in physiological saline (0.9% Baxter Healthcare Ltd., Thetford, UK) and used to assess assay cross reactivity.

### 7.3.4 Assays

The assay for plasma big ET-1<sub>[1-38]</sub> and ET-1<sub>[1-21]</sub> has been previously described [Newby *et al* 1998] with intra-assay coefficients of variability of 7.0 and 7.2% respectively and inter-assay coefficients of variability of 9.0 and 9.3% respectively. A commercially available solid phase sandwich enzyme linked immunosorbent assay for ET-1<sub>[1-31]</sub> (Immuno-Biological Laboratories Co Ltd., Tokyo, Japan) was used. This technique was performed on reconstituted samples by using anti-human ET-1<sub>[25-31]</sub> rabbit immunoglobulin G. Briefly, 100 µL of sample extract, standard or quality control along with 100 µL of ET-1<sub>[1-31]</sub> antibody, was incubated for 24 hours at 4°C. Following incubation, the wells were vigorously washed with buffer, 100 µL of labelled antibody added and incubated for a further 30 min at 37 °C. After further washing, tetramethylbenzidine buffer was added, and incubated for 30 min in the dark. Finally, 100 µL of 2 M sulphuric acid was added and the wells read using an automated plate reader at 450 nm.

### **7.3.5 Data handling and statistical analysis**

Cross reactivity was determined by calculating area under the curves for big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub>, and ET-1<sub>[1-21]</sub>, and expressed as a percentage of area under the curve for ET-1<sub>[1-31]</sub>. Intra-assay co-efficients of variation were calculated from mean values divided by standard deviation and expressed as a percentage. Plasma concentrations are expressed as mean  $\pm$  standard error of the mean. Data were examined by analysis of variance (ANOVA; Excel v5.0, Microsoft). Statistical significance was taken at the 5% level.



## 7.4 RESULTS

Patient details are shown in Table 7.1.

### 7.4.1 Recovery of ET-1<sub>[1-31]</sub>

Recovery of ET-1<sub>[1-31]</sub> during extraction was 73%. This is comparable to the extraction efficiency for big ET-1 and ET-1 [Newby *et al* 1998].

### 7.4.2 Specificity of ELISA for ET-1<sub>[1-31]</sub>

Standard curves for big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub>, and ET-1<sub>[1-21]</sub> were constructed (Figure 7.1). The cross reactivities for the assay were 3.8% for big ET-1<sub>[1-38]</sub>, 100% for ET-1<sub>[1-31]</sub>, and 0% for ET-1<sub>[1-21]</sub>. Intra-assay co-efficients of variation were 14.7, 8.8 and 6.7% at plasma ET-1<sub>[1-31]</sub> concentrations of 10, 20 and 40 pg/mL respectively.

### 7.4.3 Plasma concentrations of big ET-1, ET-1 and ET-1<sub>[1-31]</sub>

Plasma big ET-1<sub>[1-38]</sub> concentrations were elevated in patients compared with controls ( $17.1 \pm 4.4$  vs  $8.9 \pm 3.4$  pg/mL,  $p = 0.002$ ). However, there were no differences in the plasma concentrations of ET-1<sub>[1-21]</sub> ( $3.3 \pm 0.4$  vs  $3.4 \pm 0.7$  pg/mL,  $p = 0.7$ ) or ET-1<sub>[1-31]</sub> (both  $1.1 \pm 0.1$  pg/mL,  $p = 0.2$ ) (Figure 7.2).

a)

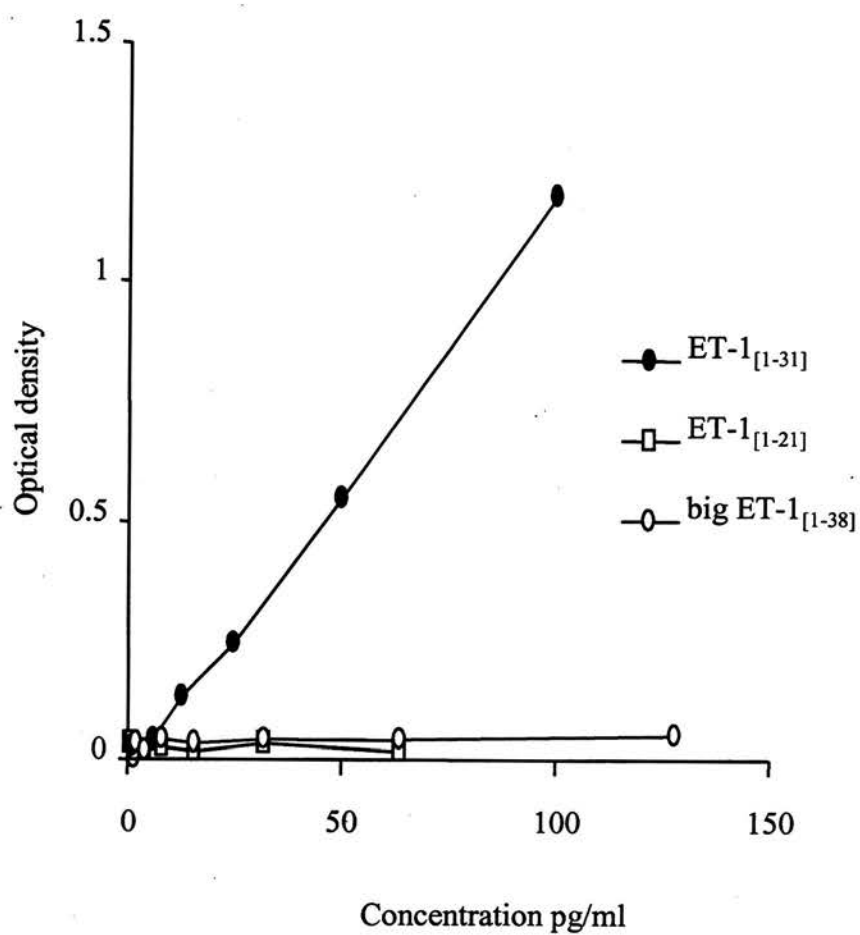
Patient	Age (years)	BMI (kgm <sup>-2</sup> )	Chol (mmol/L)	Smoker	BP (mmHg)	NYHA	Drugs
1	67	22	6.9	N	143/105	III	ASA EN BU AMIO
2	78	26	6.4	N	195/95	II	DYP BU
3	57	28	4.2	N	116/68	III	ASA EN FRU SIM DIG ISMO
4	47	26	6.1	Y	112/72	II	ASA EN FRU ISMO
5	69	29	6.1	N	121/68	II	ASA EN FRUS BIS
6	76	25	5.3	N	140/80	III	ASA EN FRU
7	80	26	5.0	Y	190/90	II	ASA LOS FRU DIG
8	77	28	4.2	Y	175/80	III	ASA CAP AMIO
9	81	23	5.5	N	120/60	II	ASA EN DIG SIM
AVE	70	26	5.5		146/80		
SEM	4	1	0.3		11/5		

b)

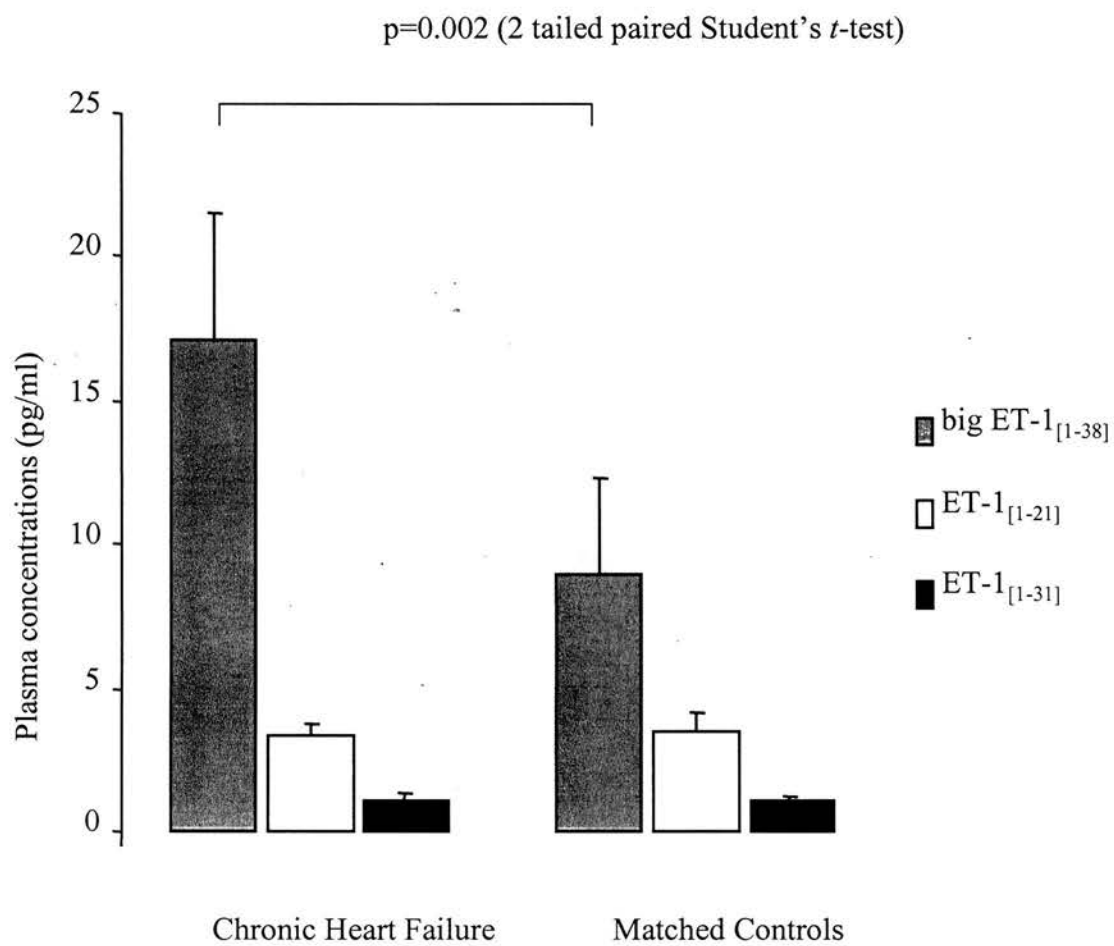
Control	Age (years)	BMI (kgm <sup>-2</sup> )	Chol (mmol/L)	Smoker	BP (mmHg)	NYHA	Drugs
1	67	26	4.8	N	124/80	NA	None
2	77	29	5.8	N	158/90	NA	None
3	73	24	4.3	N	132/88	NA	None
4	48	25	5.1	N	128/80	NA	None
5	67	26	4.7	N	145/87	NA	None
6	73	23	7.0	N	135/72	NA	None
7	81	27	5.4	N	127/64	NA	None
8	77	27	6.7	N	117/77	NA	None
9	79	29	4.4	N	103/72	NA	None
AVE	71	26	5.4		130/79		
SEM	3	1	0.3		5/3		

Table 7.1

a) Patient and b) Control Characteristics. BMI (body mass index), BP (Blood pressure), Chol (serum cholesterol). ASA = Aspirin, EN = Enalapril, BU = Bumetanide, AMIO = Amidarone, DYP = Dypyrindamol, FRU = Frusemide, SIM = Simvastatin, DIG = Digoxin, ISMO = Isosorbide Mononitrate, BIS = Bisoprolol, LOS = Losartan, CAP = Captopril.



**Figure 7.1** Standard curves of cross reactivities of big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub>, and ET-1<sub>[1-21]</sub>



**Figure 7.2** Concentrations of plasma big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub> and ET-1<sub>[1-21]</sub> in patients with chronic heart failure and matched controls.

## 7.5 DISCUSSION

This small study confirms that a new ELISA has high specificity for ET-1<sub>[1-31]</sub>, with low cross-reactivity for both ET-1<sub>[1-21]</sub> and big ET-1<sub>[1-38]</sub>. In addition, we have demonstrated, for the first time, that ET-1<sub>[1-31]</sub> immunoreactivity is present in human plasma of both CHF patients and healthy subjects. Despite elevation of the precursor peptide big ET-1<sub>[1-38]</sub>, plasma ET-1<sub>[1-31]</sub> concentrations were normal in patients with CHF.

We have demonstrated that the commercially available assay for ET-1<sub>[1-31]</sub> has a high degree of specificity for this form of endothelin compared with big ET-1<sub>[1-38]</sub> and ET-1<sub>[1-21]</sub>. The ELISA utilises an antibody that recognises the 7 amino acid carboxy terminus of ET-1<sub>[1-31]</sub>. Given that big ET-1<sub>[1-38]</sub> also shares this common sequence, it may have been anticipated that significant cross reactivity might occur. However, we report a very low level of cross reactivity and this is likely to reflect differences in immunoreactivity conferred by the tertiary structure of big ET-1<sub>[1-38]</sub> and ET-1<sub>[1-31]</sub>.

Plasma big ET-1 concentrations are elevated in patients with CHF and correlate with disease severity [Wei *et al* 1994]. Our findings are consistent with these prior data, although plasma ET-1<sub>[1-21]</sub> concentrations were not elevated. This was unexpected as plasma ET-1<sub>[1-21]</sub> concentrations are also reported to be elevated in patients with chronic heart failure [Wei *et al* 1994]. However, big ET-1<sub>[1-38]</sub> plasma concentrations are a more reliable marker of chronic heart failure and, in our study, these were increased by ~2 fold. We have also recruited stable patients who were well controlled on medical therapy. It is likely, therefore, that our patient population had

less neurohormonal activation and may have had less generation of big ET-1<sub>[1-38]</sub> than those enrolled into previous studies.

In patients with chronic heart failure, there does not appear to be a general increase in generation of ET-1<sub>[1-31]</sub>. This study does not however exclude the possibility of local increases in ET-1<sub>[1-31]</sub> production, such as within areas of inflammation. Moreover, local ET-1<sub>[1-31]</sub> generation may be specific to those tissues with increased chymase activity such as failing human myocardium [Urata *et al* 1990]. In addition, increased chymase activity is seen in mast cells at the shoulder regions of coronary atheromatous plaques [Kovanen *et al* 1995, Kaartinen *et al* 1994], and ET-1<sub>[1-31]</sub> may be implicated in the aetiology of coronary artery spasm at the time of plaque rupture. Further studies are clearly required to determine how ET-1<sub>[1-31]</sub> is processed and to identify situations where increased local ET-1<sub>[1-31]</sub> generation may occur.

It should be noted that these patients were being treated for chronic heart failure in the late 1990s, and would be considered now to be 'undertreated'. In particular the poor use of beta-blockers and the persistent hypertension, may affect the applicability of these results to more contemporary cohorts of heart failure patients.

In conclusion, this study has shown that plasma ET-1<sub>[1-31]</sub> concentrations can be measured reliably but, in contrast to big ET-1<sub>[1-38]</sub>, do not appear to be elevated in patients with heart failure. These data suggest that ET-1<sub>[1-31]</sub> is not useful as a predictive test and that generation is not sufficient to increase circulation plasma concentrations in patients with stable chronic heart failure.

## Chapter 8

### **Endothelin and its antagonists in the isolated human myocardium**

Leslie SJ, Webb DJ, Denvir MA. Endothelin-1 antagonises beta-adrenergic stimulation in human right atrial myocardium. *Journal of Basic and Clinical Cardiology* 2003;6:93-4.

## 8.1 SUMMARY

The direct effects of ET blockade on human myocardium in terms of force of contraction cannot be determined from systemic *in vivo* studies due to compensatory mechanisms previously discussed (Chapter 1). Indeed, ET-1 has previously been shown to be positively inotropic in isolated human myocardium via the ET<sub>A</sub> receptor. Beta-adrenergic agonism has positive inotropic effects, mainly via the B<sub>1</sub> receptor. These two distinct systems share a common second messenger. In membrane studies beta-agonists increase adenylate cyclase (AC) while ET-1 reduces it and inhibits isoprenaline induced AC activity. Thus, there is potential antagonism between the two systems. The aim of these experiments was to determine the direct effects of ET-1 and ET blockade on isolated human myocardium and to test the hypothesis that ET-1 can antagonise the effects of beta-adrenergic stimulation. Right atrial trabeculae were harvested from 19 beating human hearts, during initiation of cardiac bypass prior to coronary artery bypass grafting, and mounted for isometric tension experiments. ET-1 (10nM) increased force by  $12.6 \pm 5.0\%$ ,  $p=0.04$ . This was preceded by a transient non-significant decrease in force.  $-3.6 \pm 1.9\%$ ,  $p=ns$ . BQ-123 and BQ-788 had no effect on basal resting tone or force of contraction in electrically stimulated isolated human right atrial trabeculae. In addition, BQ-123 and BQ-788 did not block the inotropic effects of supra-physiological concentrations of ET-1. Isoprenaline caused an increase in force which was of faster onset and larger magnitude than that seen with ET-1  $65.7 \pm 29.2\%$ ,  $p=0.01$ . The increase in force with isoprenaline was of more rapid onset, with a maximal effect seen after 5 min. When added to maximally beta-adrenergic stimulated trabeculae, ET-1 significantly attenuated the effect of isoprenaline causing a reduction in force  $96 \pm 39\%$ ,  $p<0.01$ . These results suggest that ET-1 is positively inotropic but does not contribute to resting inotropic effect in the



human myocardium and that ET-1 functionally antagonises the effects of beta-adrenergic stimulation. This may have important consequences in conditions where both systems are up regulated. Beta-blockade has recently been shown to have important prognostic benefits in patients following myocardial infarction and in patients with CHF. ET-1 is also up regulated in these conditions and may provide physiological antagonism to catecholamine over-stimulation.

## 8.2 BACKGROUND

Plasma concentrations of endothelin-1 (ET-1) are elevated in chronic heart failure (CHF) and correlate with a poor prognosis [Wei *et al* 1994]. Short-term systemic ET blockade in CHF patients causes potentially beneficial haemodynamic effects; increasing cardiac output and reducing vascular resistance [Kiowski *et al* 2001]. However, the initial results from longer term clinical trials have been disappointing. In the ENABLE and EARTH clinical trials preliminary reports indicate that there was no major benefit when an endothelin receptor antagonist (ETRA) was added to conventional heart failure therapy.

Beta-blockers improve mortality in CHF patients and while the mechanisms for these benefits are not fully understood they may involve  $\beta_1$ -adrenoreceptor upregulation.  $\beta_1$ -adrenergic stimulation increases adenylate cyclase (AC), increasing cAMP, activating protein kinase A, resulting in phosphorylation of several intracellular proteins ultimately leading to an increase in intracellular calcium.

In systemic studies, administration of ET-1 reduces cardiac output, probably due to a combination of coronary artery constriction, reducing myocardial blood supply, and systemic vasoconstriction increasing systemic vascular resistance and thus after-load [Kiely *et al* 1997]. However, ET-1 increases force of contraction in isolated myocardium, [Beyer *et al* 1995, Meyer *et al* 1996, Saetrum Opgaard *et al* 2000, Dhein *et al* 2000], and in isolated human myocardium  $ET_A$  receptor antagonists have been shown to inhibit ET-1 mediated inotropy [Meyer *et al* 1996, Dhein *et al* 2000, Saetrum Opgaard *et al* 2000] although the effects of  $ET_B$  receptor antagonists have been less consistent showing no effect [Dhein *et al* 2000] or blockade of ET inotropy

[Saetrum Opgaard *et al* 2000]. The exact mechanism by which ET-1 increases cardiac contractility is uncertain, however there are several intracellular changes which can increase force of contraction such as increasing calcium availability, increasing myofilament sensitivity to calcium and increase of intracellular pH [Meyer *et al* 1996]. Binding to the ETR results in activation of a number of intracellular signalling processes, it has been suggested than more than one second messenger signalling system may be stimulated simultaneously depending on the cell type and level of expression of G protein subtypes. G protein subtypes coupled to ETRs include G<sub>q</sub>, G<sub>11</sub>, G<sub>s</sub> and G<sub>12</sub> [Takigawa *et al* 1995] (Figure 1.4). There are many downstream signal transduction pathways including nitric oxide synthase, adenylate cyclase, protein kinase C, and phospholipase A<sub>2</sub>, C and D, ion channels such as calcium and chloride channels and ion transporters. Therefore the effects of binding to ETRs will also depend on which second messenger pathways are activated and this will change within and between cell types. In addition, many of these second messenger pathways are shared with other neuroendocrine systems and therefore post receptor cross talk may influence responses. One such potential interaction is with the beta-adrenergic system. Beta-adrenergic stimulation results in activation of adenylate cyclase (AC), resulting in increased intracellular cAMP, activation protein kinase A causing phosphorylation of several intracellular proteins. This phosphorylation has the effect of decreasing myofilament sensitivity for calcium but increasing intracellular calcium by stimulation of sarcoplasmic reticular release as well as increased influx of extracellular calcium. Membrane studies have demonstrated than isoprenaline (ISO), a selective beta-1 receptor agonist, increases AC while ET inhibits its activity and can inhibit ISO stimulated increases in AC [Vogelsang *et al* 1994, Ponicke *et al* 1998]. Thus, there is a potential antagonistic interaction between these two systems.

The aim of these experiments was to determine the direct effects of ET-1 and ET blockade and to examine the hypothesis that ET-1 antagonises the effects of beta-adrenergic stimulation in isolated human myocardium thus avoiding confounding compensatory mechanisms.

## **8.3 METHODS**

### **8.3.1 Patients**

Nineteen patients undergoing coronary artery bypass grafting for coronary artery disease requiring cardiopulmonary bypass participated with the approval of the Lothian Research Ethics Committee and the written informed consent of each subject. The characteristics of the patients are described in Table 8.1. This group represents the typical patient undergoing coronary artery bypass grafting.

### **8.3.2 Study Design**

Isometrically twitching right atrial trabeculae were allowed to stabilise for 1 hour before starting the experiment. Any preparations with an unstable baseline or 'slow' twitch were discarded. Twelve stable trabeculae were exposed to either BQ-123 (1mM) or BQ-788 (1mM). Responses to ET-1 were then determined where paired data were possible. In a separate series of experiments, stable trabeculae were exposed to either isoprenaline (10mM), ET-1 (10mM) or ISO followed by ET-1 at the same concentration.

### **8.3.3 Myocardial trabeculae preparation**

Myocardial trabeculae were collected and processed as previously described in Chapter 2. Free running trabeculae were mounted for electrical stimulation at 1Hz at a voltage ~10% above threshold with rectangular pulses of 10 ms duration and allowed to stabilise for 1 hour in a vertical 7ml chamber (World Precision Instruments, UK) containing modified Tyrodes (NaCl 130, KCl 5.4, NaHPO<sub>4</sub> 0.56, MgCl<sub>6</sub>H<sub>2</sub>O 3.5,

CaCl<sub>2</sub> 2, Glucose 10, HEPES 5(mM)) corrected to pH 7.4 with NaOH and continuously bubbled with 100% O<sub>2</sub> at 35°C.

#### **8.3.4 Solutions**

Physiological solution had the following composition (mM); NaCl 130, KCl 5.4, NaHPO<sub>4</sub> 0.56, MgCl<sub>6</sub>H<sub>2</sub>O 3.5, CaCl 2, Glucose 10, HEPES 5. This was corrected to pH 7.4 with NaOH. Cardioplegic solution consisted of physiological solution with the addition of the cardioplegic agent 2,3 butane-dione-monoxime 30mM.

#### **8.3.5 Drugs**

ET-1, BQ-123 and BQ-788 (Neosystem SNPE England, Croydon, UK) were reconstituted following the manufacturers instructions with saline 0.9% (Baxter Healthcare Ltd, Thetford, UK) and diluted in physiological solution. Isoprenaline (Sigma-Aldrich Chemicals, Poole, UK) was dissolved in physiological solution with ascorbic acid (1mM) to reduce oxidation. Solutions were kept at room temperature and protected from light during the experiment. All other chemicals were of analytical grade (Merck, Magna Park, Leicestershire, UK).

#### **8.3.6 Justification for drug concentration**

During systemic dosing studies, the total dose of BQ-123 and BQ-788 administered in the study was 1000 and 300 nmol/min for 15 min. Assuming a circulating blood volume of 3L this would give approximate plasma concentrations of 5 and 1.5 µM respectively. Tissue concentrations are likely to be much lower than this, however, it is not possible to accurately estimate tissue concentrations of drug in human myocardium as this will depend on several, difficult to determine variables, such as

plasma concentrations in coronary arteries, tissue perfusion and permeability. Previous researchers have demonstrated effects of ET antagonists *in vitro*, at concentrations of 0.1  $\mu\text{M}$  and therefore, in order to determine whether endothelin antagonists would have any direct cardiac effect we exposed isolated human myocardial trabeculae to concentrations of 1  $\mu\text{M}$ , 10 fold higher than previous researcher have employed and although these concentrations are lower than the maximal possible concentration in the systemic studies they are likely to be significantly higher than physiological tissue concentrations.

### **8.3.7 Data analysis and statistics**

Isometric twitches were detected by a force transducer (FORT 10, World Precision Instruments, Stevenage, UK) and amplified (Transbridge<sup>TM</sup>). Analogue voltage output was processed by a MacLab<sup>®</sup> analogue-to-digital converter and Chart v3.3 software (AD Instruments, New Zealand) and recorded onto a Macintosh Classic II computer (Power Macintosh 7200/90, Apple Computers Inc, Cupertino, USA). Statistical difference was tested by 2 tailed, paired Student's *t*-test (Excel v5.0, Microsoft). A value of  $p < 0.05$  was considered to be statistically significant. All values are expressed as mean  $\pm$  SEM.

**A**

Age (years)	59 ± 2
Sex	11 Male : 1 Female
Smoking	1 non : 11 ex
LV dysfunction	8 preserved 1 mild dysfunction 1 moderate dysfunction 2 not reported
Heart Rate (bpm)	60 ± 2
Systolic BP (mmHg)	135 ± 4
Diastolic BP (mmHg)	66 ± 2
Urea (mmol/L)	5.9 ± 2.2
Creatinine (μmol/L)	87 ± 2
Beta-blocker	11/12
Aspirin	12 (stopped for 5 days)

**B**

Age (years)	65 ± 3
Sex	7 male
Smoking	2 non : 5 ex
LV dysfunction	5 preserved 2 moderate dysfunction
Systolic BP (mmHg)	135 ± 4
Diastolic BP (mmHg)	66 ± 2
Urea (mmol/L)	6.7 ± 0.6
Creatinine (μmol/L)	102 ± 6
Beta-blocker	7/7
Aspirin	5/5 (stopped for 5 days)

**Table 8.1** Patient characteristics

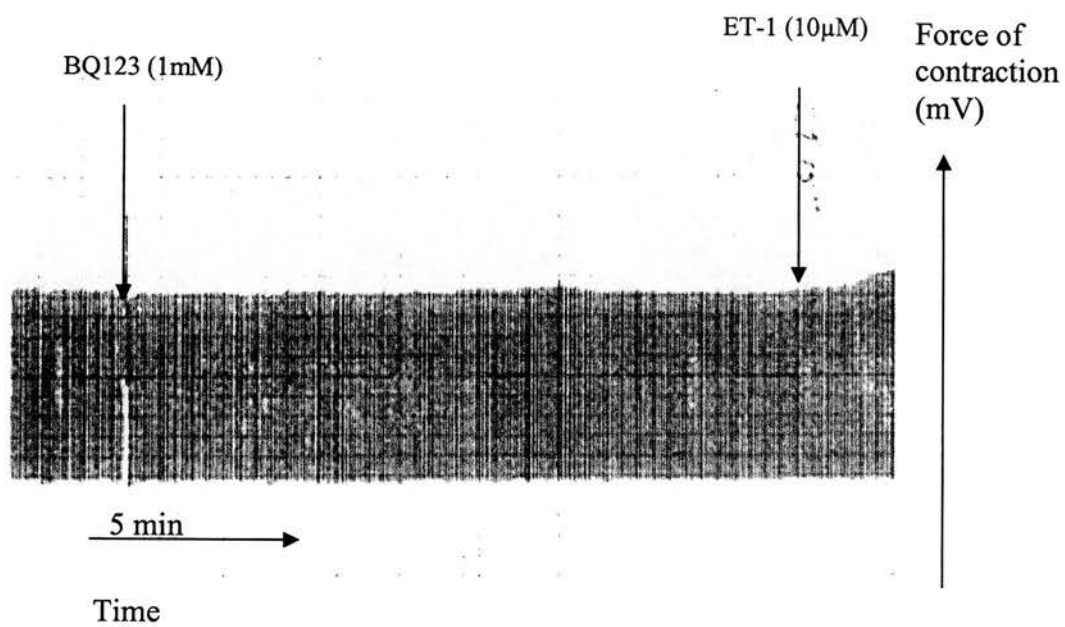


## 8.4 RESULT

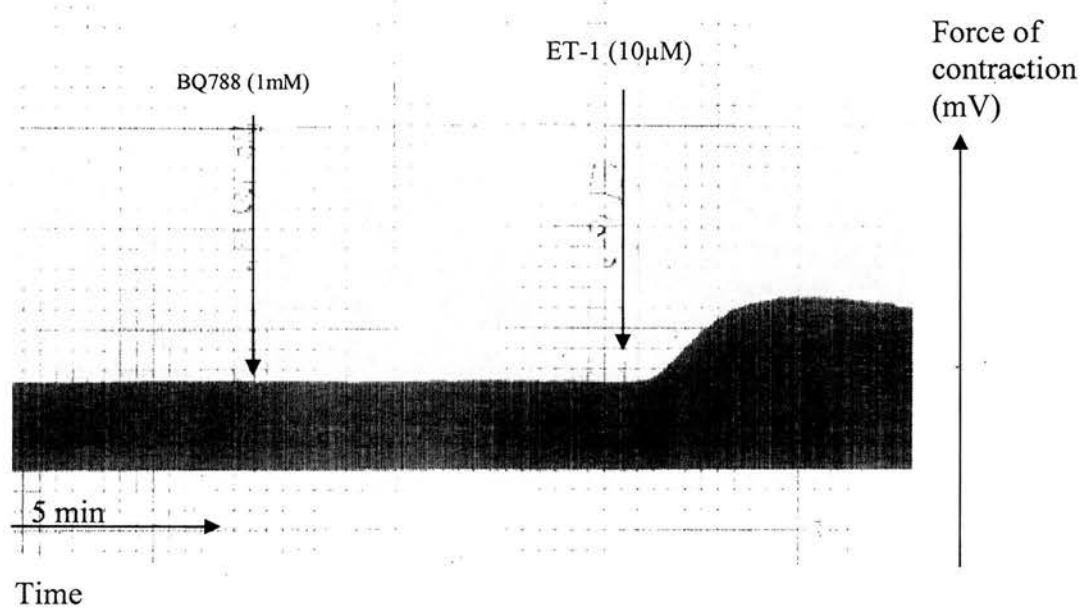
BQ-123 (1mM) caused no increase in force of contraction vs placebo from baseline (n=7)(Figure 8.1). BQ-788 (1mM) caused no change in force of contraction vs placebo from baseline (n=2) (Figure 8.2). Neither BQ-123 nor BQ-788 appeared to block responses to supra-physiological concentrations of ET-1 in our preparations (Figures 8.3 and 8.4).

Compared with vehicle, ET-1 (10nM) increased force by  $12.6 \pm 5.0\%$ ,  $p=0.04$ . (Figure 8.5). Compared with vehicle, isoprenaline caused an increase in force which was of faster onset and larger magnitude than the inotropy seen with ET-1 ( $65.7 \pm 29.2\%$ ,  $p=0.01$ ). The increase in force with isoprenaline was of more rapid onset, with a maximal effect seen after 5 min. When added to maximally beta-adrenergic stimulated trabeculae, ET-1 significantly attenuated the effect of isoprenaline causing a reduction in force  $96 \pm 39\%$ ,  $p<0.01$  (Figure 8.6).

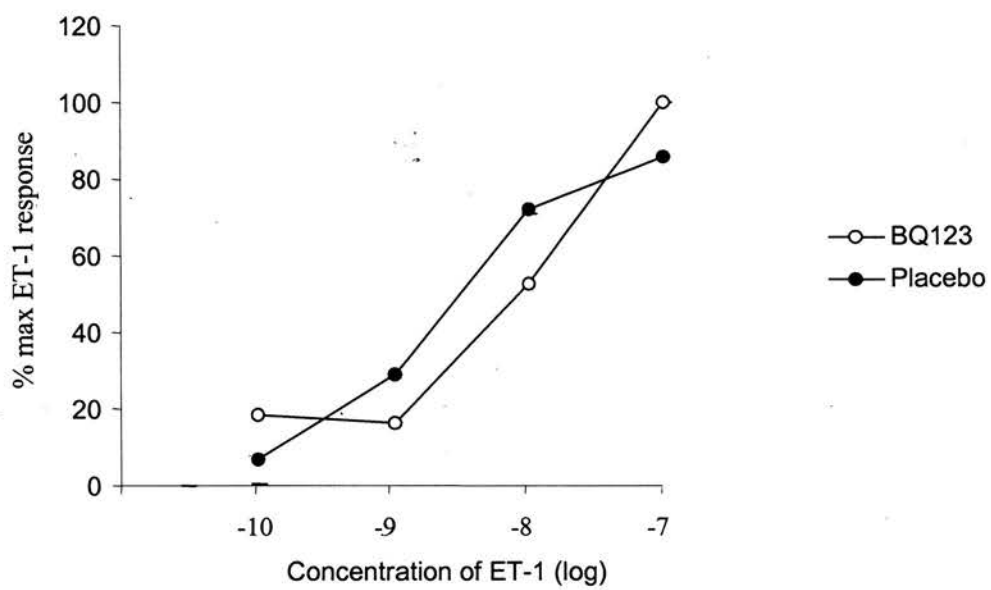
ET-1 resulted in an increase in twitch parameters with a trend towards an increase in time to peak ( $160 \pm 20$  to  $216 \pm 33$  ms,  $p = \text{ns}$ ) and an increase in both  $RT_{50}$  ( $106 \pm 11$  to  $129 \pm 14$  ms,  $p < 0.05$ ) and  $RT_{95}$  ( $221 \pm 24$  to  $251 \pm 21$  ms,  $p < 0.05$ ). Isoprenaline caused a trend towards a shortening of the twitch parameters, time to peak ( $180 \pm 40$  to  $163 \pm 30$  ms,  $p = \text{ns}$ ),  $RT_{50}$  ( $113 \pm 7$  to  $111 \pm 12$ ,  $p = \text{ns}$ ) and  $RT_{95}$  ( $238 \pm 10$  to  $228 \pm 12$ ,  $p = \text{ns}$ )(Table 8.2 and Figure 8.7).



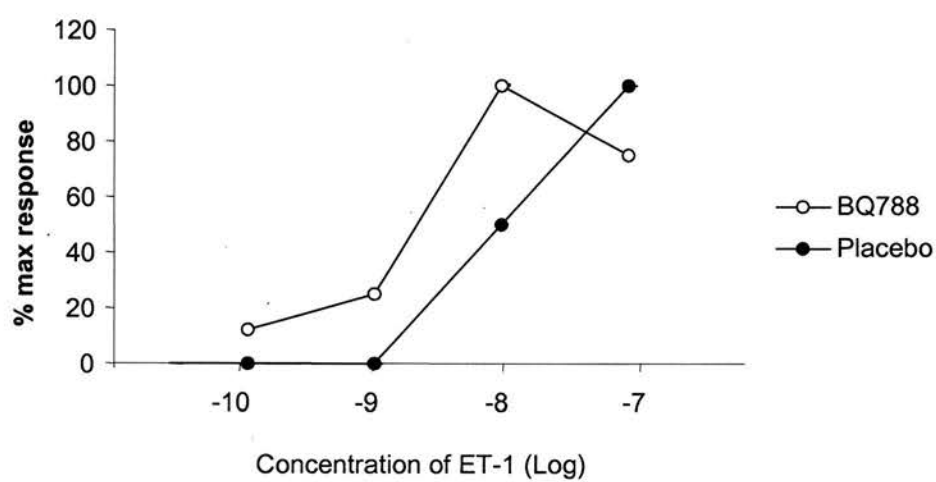
**Figure 8.1** Effect of BQ123 on basal force of contraction (actual tracing)



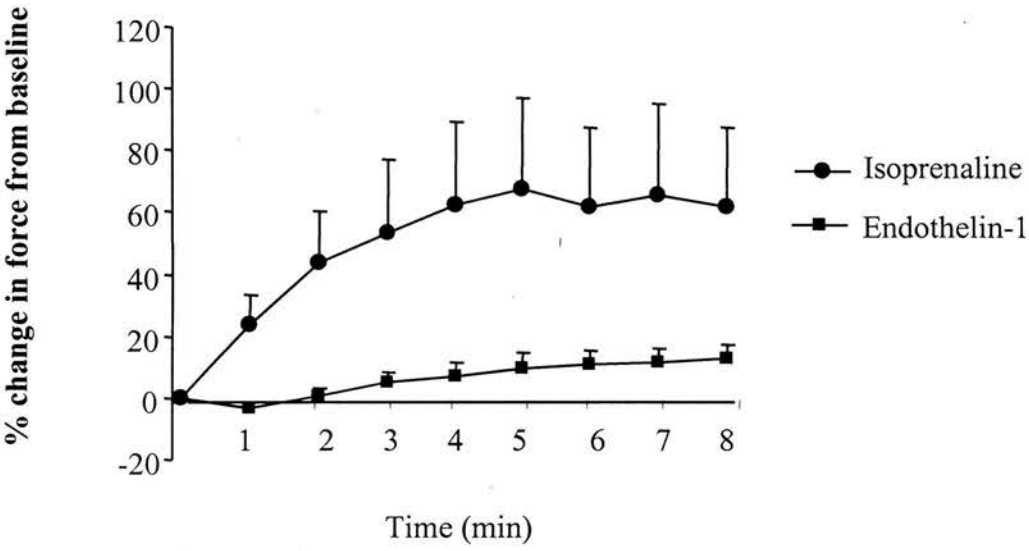
**Figure 8.2** Effect of BQ123 on basal force of contraction (actual tracing)



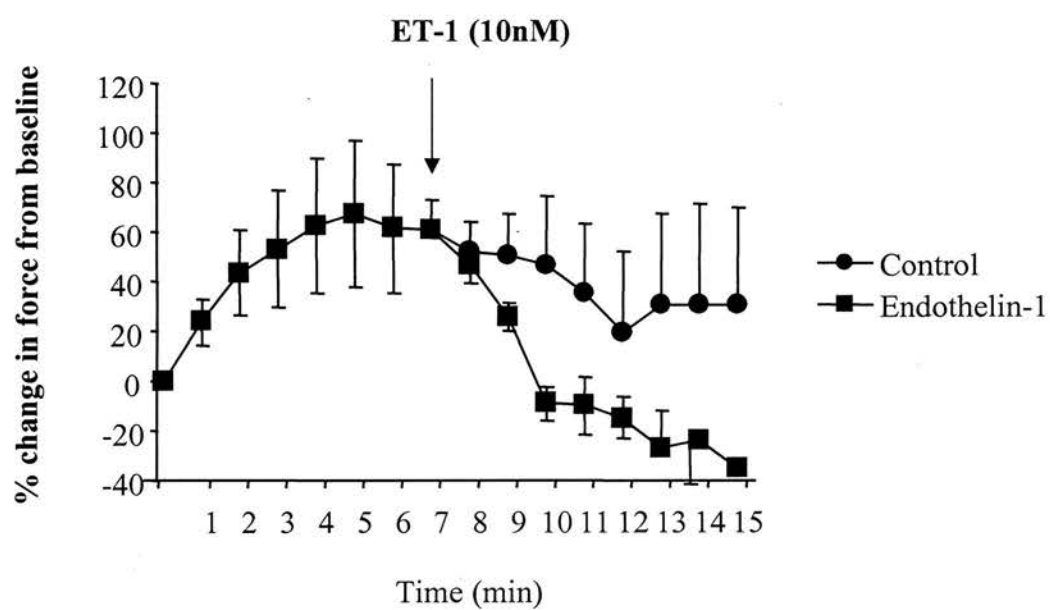
**Figure 8.3** Effect of BQ123 on ET-1 inotropy



**Figure 8.4** Effect of BQ788 on ET-1 inotropy



**Figure 8.5** Effect of ET-1 and isoprenaline on force of contraction

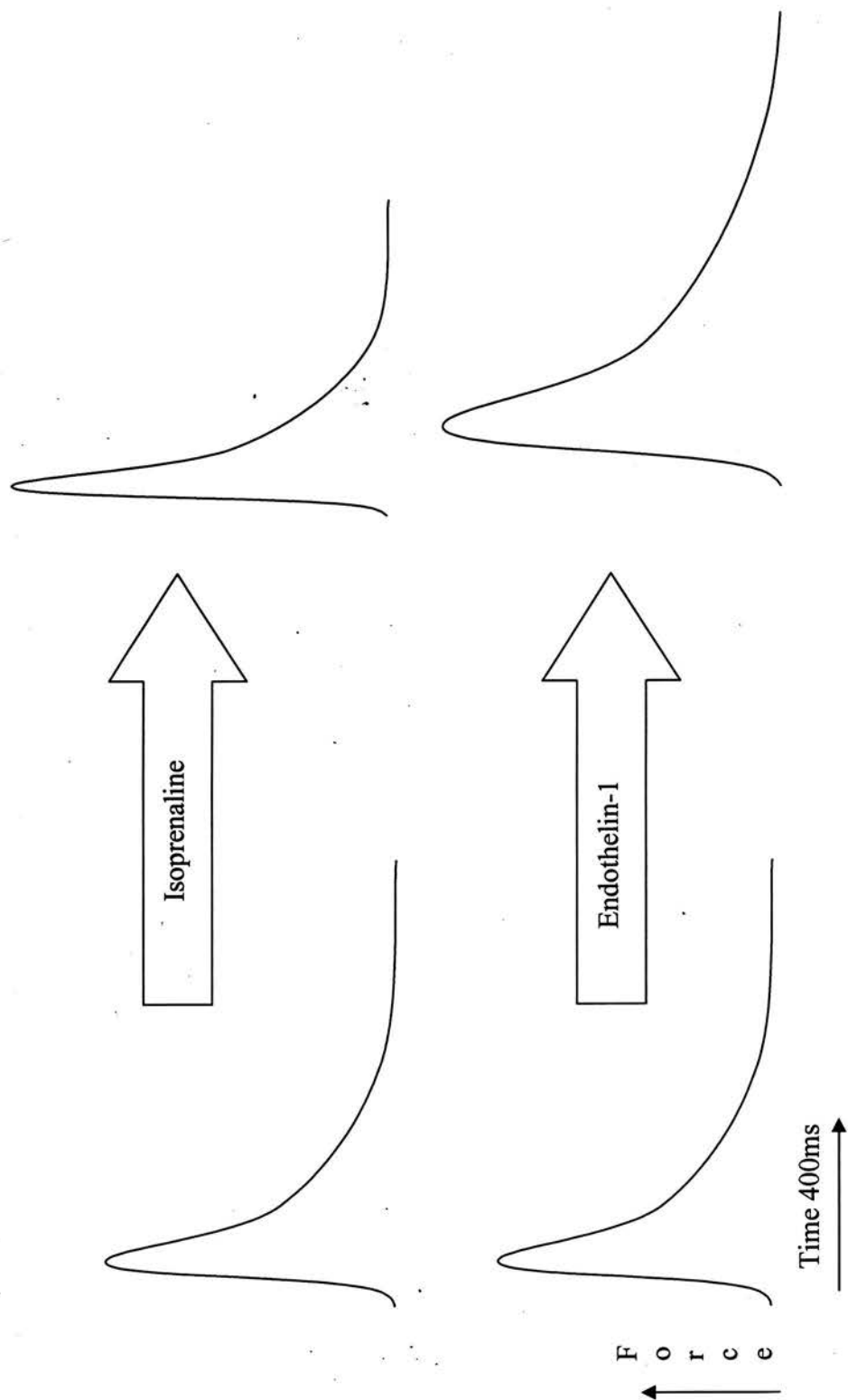


**Figure 8.6** Effect of endothelin on maximally isoprenaline stimulated myocardium

<b>Time</b>	<b>Baseline</b>	<b>ISO (10 nM)</b>	<b>Baseline</b>	<b>ET-1 (10nM)</b>
<b>TTP</b>	180 ± 40	163 ± 30	160 ± 20	216 ± 33
<b>RT<sub>50</sub></b>	113 ± 7	111 ± 12	106 ± 11	129 ± 14*
<b>RT<sub>95</sub></b>	238 ± 10	228 ± 12	221 ± 24	251 ± 21*
<b>Force</b>	362 ± 69	496 ± 100*	348 ± 67	389 ± 89*

**Table 8.2** Influence of isoprenaline (ISO) and endothelin-1 (ET-1) on twitch parameters; time to peak (TTP), RT<sub>50</sub>, RT<sub>95</sub>, and force of contraction (Force). (\* = P<0.05)





**Figure 8.7** Diagram of the effects of isoprenaline and endothelin-1 on twitch parameters (for values see Table 8.2)

## 8.5 DISCUSSION

This study has confirmed that ET-1 and beta-adrenergic stimulation increase force of contraction in isolated human myocardium. ET antagonism does not affect basal tone in isolated myocardium suggesting that there is no resting ET-1 mediated inotropy. ET-1 attenuates the inotropic effects of beta-adrenergic stimulation with isoprenaline.

### 8.5.1 Effects of ET antagonists on myocardial force of contraction

Both ET<sub>A</sub> and ET<sub>B</sub> receptors are present in human myocardium [Ponicke *et al* 1998]. However, distribution of the receptor type and number is not uniform and there may be differences in specific pathological conditions. In situ hybridization studies have demonstrated both receptor types in atrial and ventricular myocardium, atrioventricular conducting system and the endocardial cell [Molenaar *et al* 1992]. The ratio of ET<sub>A</sub> to ET<sub>B</sub> receptors in terms of number is approximately 60:40% [Peter & Davenport 1996]. However, overall, receptor density is 1.5 to 2 fold higher in the atrium compared to the ventricle [Ponicke *et al* 1998]. In addition, a higher proportion of ET<sub>B</sub> receptors are found in the AV node and the penetrating bundles of His compared with the surrounding myocardium [Molenaar *et al* 1992] suggesting involvement of the ET<sub>B</sub> receptor in the neural control of the heart

In isolated human myocardium, ET<sub>A</sub> receptor antagonist have been shown to inhibit ET-1 mediated inotropy [Meyer *et al* 1996, Dhein *et al* 2000, Saetrum Opgaard *et al* 2000] although the effects of ET<sub>B</sub> receptor antagonists have been less consistent showing no effect [Dhein *et al* 2000] or blockade of ET inotropy [Saetrum Opgaard *et al* 2000]. The effects of local studies on isolated myocardium contrast with the effect on cardiac output seen in systemic studies.

In these experiments we have demonstrated that neither the selective ET<sub>A</sub> antagonist, BQ-123, or the selective ET<sub>B</sub> antagonist, BQ-788 has an effect on force of contraction on isometrically twitching human myocardium. In addition, pre-treatment with ET antagonists did not antagonise the effects of ET-1. This was unexpected. As discussed above, other groups have demonstrated that pre-treatment with ET antagonists can reduce the potency of ET-1 although responses to maximal doses ( $E_{\max}$ ) are not reduced probably due to competitive binding at receptor sites.

There are several possibilities why ET antagonists did not appear to antagonise ET-1 responses in this study. Firstly the dose used may have been inadequate. However, this is unlikely as the doses used are comparable to estimated plasma concentrations and are higher than the concentrations of antagonists used by other researchers. It is possible that the antagonists may have been degraded, however drugs were prepared in keeping with the manufacturers instruction and stored at  $-80^{\circ}\text{C}$ . In addition, it is our experience that both BQ-123 and BQ-788 are relatively stable compounds even at room temperature. In a separate experiment (data not shown), the degradation of BQ-788 was determined after being kept for 24 hours at room temperature ( $18-20^{\circ}\text{C}$ ). These data from HPLC were compared with clinical grade BQ-788 and found to be similar and there was no discernable degradation as measured by HPLC. However, the biological activity was not measured and therefore we cannot be certain that the batch of BQ-788 was biologically inert.

Thus, it would appear that in our preparations ET antagonism has no effect on basal resting tone and, therefore that the effects of ET blockade on cardiac output are

primarily a result of effects on the peripheral or coronary vasculature rather than direct effects on the myocardium. It should be noted that these data were gained in patients with a primary diagnosis of coronary artery disease rather than heart failure. In addition, they were performed on atrial tissue where clearly the effects on ventricular tissue are more important with regard to cardiac pump function.

### **8.5.2 ET-1 and beta-adrenergic interaction**

Both ET-1 and isoprenaline increased isometric force in human RA myocardium. ET-1 increased force following a transient negative inotropic response, and although this has been described by other groups [Ponicke *et al* 1998, Dhein *et al* 2000] the mechanism remains unclear. In addition ET-1 caused a significant increase in twitch parameter times, consistent with an increase in myofilament sensitivity. This confirms results from previous studies where there are increases in myofilament sensitivity via activation of PKC and activation of the sarcolemmal  $\text{Na}^+/\text{H}^+$  exchanger [Meyer *et al* 1996]. ET-1 has also been shown to increase intracellular  $\text{Ca}^{2+}$ . The mechanisms behind this are unclear but increases in intracellular  $\text{Na}^+$  will reduce  $\text{Ca}^{2+}$  extrusion by the sarcolemmal  $\text{Na}^+/\text{Ca}^{2+}$  exchanger thus increasing intracellular  $\text{Ca}^{2+}$  and force of contraction [Meyer *et al* 1996].

In these experiments, a transient negative inotropic effect was seen following ET-1 administration although it failed to reach statistical significance. This finding is consistent with work from previous groups although the mechanism is not clear [Vogelsang *et al* 1994, Dhein *et al* 2000].

Isoprenaline caused an increase in force of contraction that was of faster onset and larger magnitude than that seen with ET-1 and caused a non-significant reduction in twitch duration consistent with reducing myofilament sensitivity. This result was expected as beta-adrenergic stimulation results in an increase in intracellular  $\text{Ca}^{2+}$  secondary to both sarcolemmal channel opening and release from intracellular stores, resulting in increases in force of contraction.

We have observed, for the first time, that ET-1 functionally attenuates the inotropic effects of isoprenaline in human myocardium. This reduction in force was observed after maximal stimulation of the beta-adrenergic system. The underlying mechanism is likely to be interaction at the level of AC as seen in isolated membrane studies where ET-1 was shown to inhibit the effect of  $\beta_1$ -adrenoreceptor activation on this enzyme.

Unfortunately the apparatus to measure intracellular  $\text{Ca}^{2+}$  was not available at the time of performing these experiments and therefore no comment can be made about the effect of this antagonism on intracellular  $\text{Ca}^{2+}$ . However, it would be expected that intracellular  $\text{Ca}^{2+}$  transients may be decreased by the effects of ET-1 as antagonism at the level of adenylate cyclase is likely to have a more profound effect on intracellular  $\text{Ca}^{2+}$  through sarcolemmal  $\text{Ca}^{2+}$  channels and sarcoplasmic reticular release than the indirect  $\text{Ca}^{2+}$  increases as a result of activation of the  $\text{Na}^+/\text{H}^+$  exchanger in response to ET-1.

These results suggest that, in the human myocardium, ET-1 functionally antagonises the effects of beta-adrenergic stimulation. However, care should be taken when

interpreting these results as supra-physiological concentrations of agonists were used, although, it appears that there is functional antagonism between these two hormone systems, presumably at the level of receptor, or post receptor cross-talk.

This study has some limitations in that we were unable to study normal human ventricular myocardium due to a lack of suitable samples. Previous isolated membrane studies have confirmed differences between atrial and ventricular tissue so ET-1 may not inhibit AC in ventricular tissue [Ponick *et al* 1998]. In addition, the concentrations of ET-1 used were higher than those in the plasma of CHF patients but may be similar to those occurring at a local level in myocardium.

Nevertheless, the results of this study may have important consequences in conditions such as CHF where both ET-1 and the beta-adrenergic systems are up-regulated and drugs blocking their actions are being developed. Catecholamine release and therefore beta-adrenergic stimulation is increased in CHF and also in episodes of acute heart failure and acute coronary syndromes. This can lead to increases in arrhythmias and sudden cardiac death, the most common cause of death in patients with CHF. In CHF there is a reduced responsiveness to beta-agonists, probably due to combination of reduced receptor number and post receptor changes. It could be that some of these changes are due to an interaction with the ET system which is also up-regulated in CHF. The importance of this interaction may be that ET-1 while a marker of disease severity in CHF actually protects against catecholamine stimulation. It is known that catecholamine blockade with drugs such as beta-blockers confers mortality and morbidity benefits and with the introduction of ETAs for the treatment of CHF the physiological and patho-physiological interactions between these two systems is of

importance. Indeed, the first trials of endothelin antagonist in patients with heart failure, many of whom were on beta blocker treatment was negative, demonstrating no mortality benefit [Coletta *et al* 2002, Luscher *et al* 2002].

Clearly these experiments require further investigation but are beyond the scope of simple isometric force contraction experiments.

Note: Although an attempt to gain ventricular tissue was made by the author, only 4 explanted hearts were collected before the transplant program in Glasgow was temporarily suspended. Only 2 of these were suitable for experiments. There were logistical and geographical difficulties in obtaining hearts from Newcastle for trabecular experiments.

## **Chapter 9**

### **The effect of plasma lipid lowering therapy on resistance vessel tone and response to endothelin antagonism**

**Leslie SJ**, Spratt JC, Attina T, Denvir MA, Webb DJ. The effect of cerivastatin on vascular responses to endothelin antagonism. *J Cardiovasc Pharmacol* 2004;44:S410-412.



## 9.1 SUMMARY

Endothelin (ET) blocking drugs have been shown to have beneficial effects mediated at least in part via the nitric oxide (NO) system. Hypercholesterolaemia is associated with vascular dysfunction including increased arterial stiffness and impaired NO mediated vasodilatation. Treatment with HMG CoA reductase inhibitors (statins) has proven mortality benefits in a range of patient populations. Subjects (n=5) received either placebo or statin (cerivastatin 400mcg) for an 8 week period in a double-blind placebo-controlled cross-over study. Cerivastatin reduced total plasma cholesterol compared with baseline by 27 % ( $5.4 \pm 0.4$  vs  $7.3 \pm 0.4$  mmol/L,  $p = 0.04$ ). Compared with placebo, cerivastatin caused a trend towards an increase in forearm blood flow (FBF) in response to selective ET<sub>A</sub> ( $18.0 \pm 7.2$  vs  $52.0 \pm 19.0$  %,  $p = 0.06$ ). Selective ET<sub>B</sub> receptor blockade reduced FBF with no difference between placebo and cerivastatin therapy ( $-11.0 \pm 3.9$  vs  $-13.0 \pm 3.6$ %,  $p = 0.9$ ). Dual ET<sub>A/B</sub> receptor blockade increased FBF with no difference between placebo and cerivastatin therapy ( $39.8 \pm 13.4$  vs  $42.4 \pm 19.0$  %,  $p = 0.7$ ). In conclusion, statin therapy may decrease large artery stiffness and increase in the vasodilating effects of ET<sub>A</sub> receptor blockade.

## 9.2 BACKGROUND

ET-1 causes vasoconstriction via the ET<sub>A</sub> and ET<sub>B</sub> receptors located on vascular smooth muscle cells and a prostanoid and nitric oxide (NO) mediated vasodilatation via the ET<sub>B</sub> receptor on endothelial cells. Thus, ET<sub>A</sub> receptor blockade causes vasodilatation while the effects of ET<sub>B</sub> receptor blockade will depend on the balance between vasodilating and vasoconstricting effects at different sites.

In healthy subjects, brachial artery administration of BQ-123, a selective ET<sub>A</sub> antagonist, results in a forearm vasodilation of ~40% [Haynes *et al* 1994b, Berrazueta *et al* 1997, Verhaar *et al* 1998], whereas administration of BQ-788, a selective ET<sub>B</sub> antagonist, causes a modest vasoconstriction of ~10% [Verhaar *et al* 1998]. If the mechanism of the vasodilation is NO mediated, then in an NO-deficient state the dilatation to ET-1 at the endothelial ET<sub>B</sub> receptor may be reduced, with less vasoconstriction to BQ-788. Many studies have previously demonstrated evidence of endothelial dysfunction and a NO deficient state in patients with hypercholesterolaemia [Creager *et al* 1990, Celemajer *et al* 1992, Chowienczyk *et al* 1993]. Hypercholesterolaemia is also associated with a reduction in vascular compliance and a reduced response to vasoactive therapies such as nitrates. Some of these responses can be improved following lipid lowering therapy [Stroes *et al* 1995, O'Driscoll *et al* 1997]. In addition, it has been clearly demonstrated that reduction of plasma cholesterol reduces mortality in several patient groups [Scandinavian Simvastatin Survival Study Group 1994, Shepherd *et al* 1995].

The aim of this study was to examine whether treatment of hypercholesterolaemia with an HMG CoA reductase inhibitor (cerivastatin) could increase vascular compliance and improve vasodilator responses to ET antagonists.

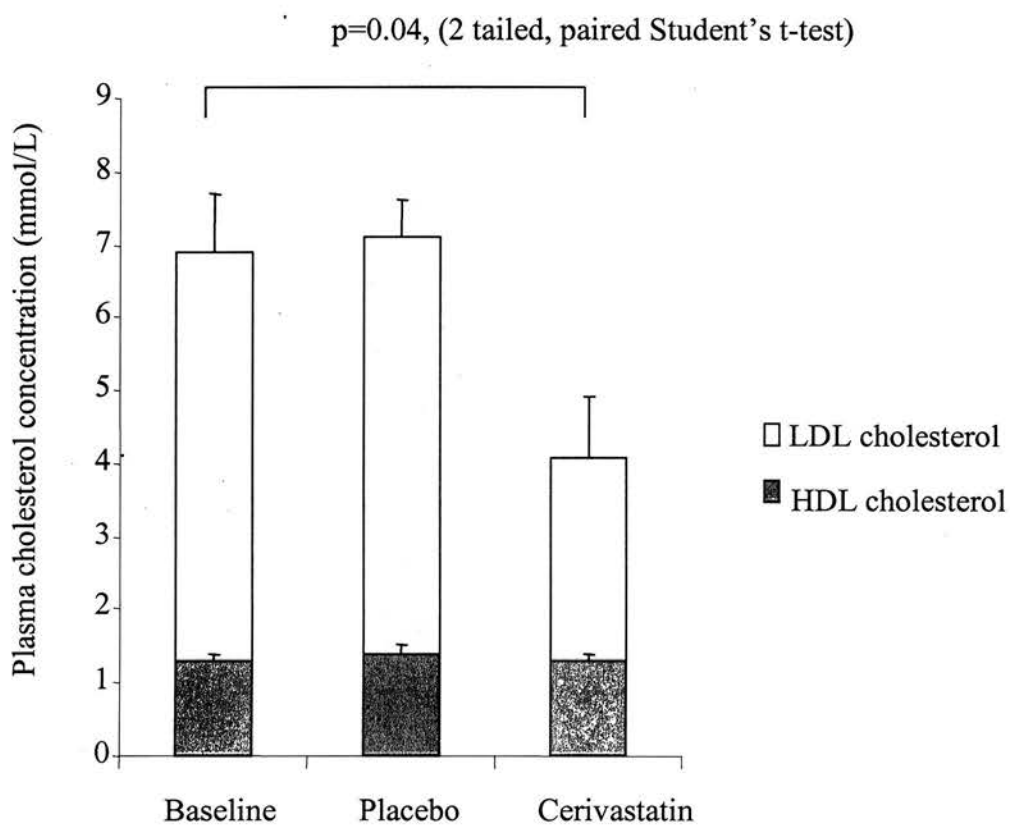
### 9.3 MATERIALS AND METHODS

Five non-smoking, otherwise healthy, hypercholesterolaemic subjects were studied. Subjects were rejected if they had diabetes mellitus, renal impairment (creatinine; men > 180 mmol/l, women > 160 mmol/l), a 10-year coronary heart disease risk of > 30%, a history of severe allergic reaction, a systolic blood pressure > 180 or < 90 mmHg or were taking any regular medications. The study was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. Written informed consent was obtained from each subject before entry into the study. Subjects attended fasted for each study visit which was performed in a quiet, draught free, temperature controlled room (22-24 °C). The technique of venous occlusion plethysmography to measure forearm blood flow [Benjamin 1995] and pulse wave analysis to measure augmentation index and arterial stiffness [Wilkinson 1998] have been described in detail elsewhere.

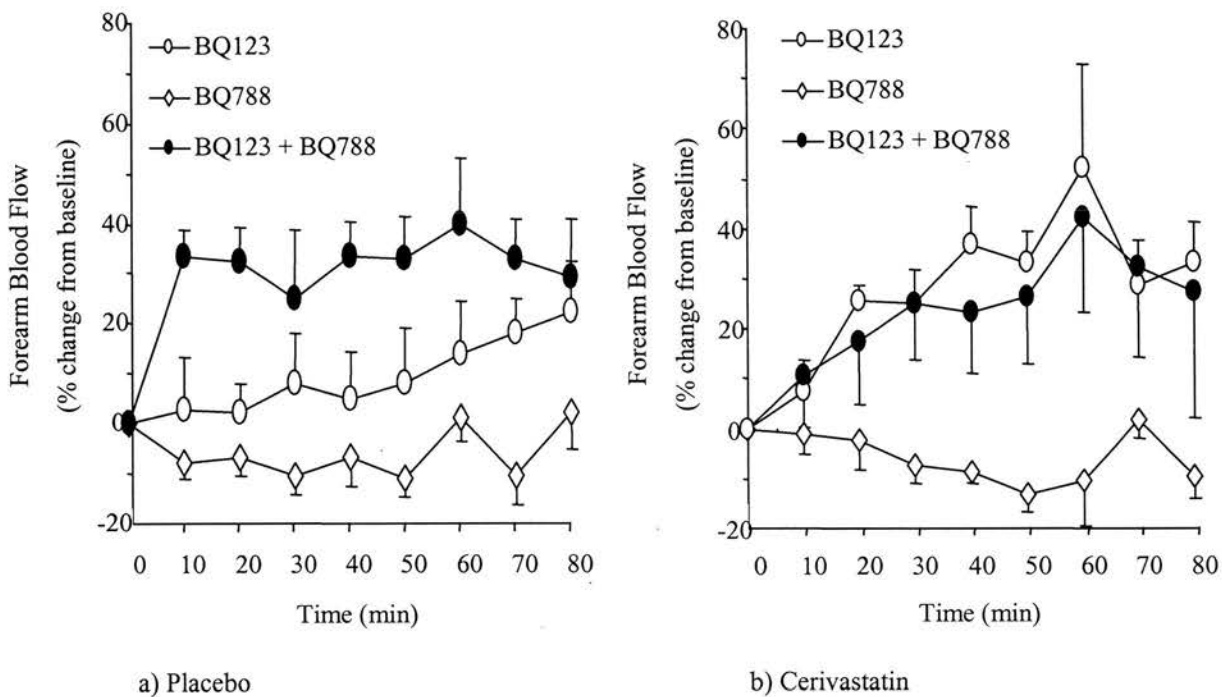
Subjects received, in a randomised double blind cross-over manner, 8 weeks treatment with either sucrose placebo or cerivastatin 800 µg nocte (Bayer AG). BQ123 and BQ788 (Clinalfa AG, Laufelfingen, Switzerland) were diluted in 0.9% saline (Baxter Healthcare Ltd, Thetford, UK) and infused via an intra-arterial needle over 80 min. Subjects received BQ123 (10 mol/min), BQ-788 (1 mmol/min) or both in a random order by 80 min intra-arterial infusion at weeks 6, 7 and 8 and at weeks 14, 15 and 16. The order of intra-arterial drug infusion at the end of each treatment period was randomised and kept the same within patients, i.e the order of ET antagonist forearm studies was consistent between treatment periods. Pulse wave analysis was performed and venous blood samples taken at weeks 0, 8 and 16. Data were examined by Student's *t*-test (Excel v5.0, Microsoft). Results are expressed as mean ± SEM. Forearm blood flow and pulse wave analysis results are expressed as percentage change from baseline. Statistical significance was taken at the 5% level.

## 9.4 RESULTS

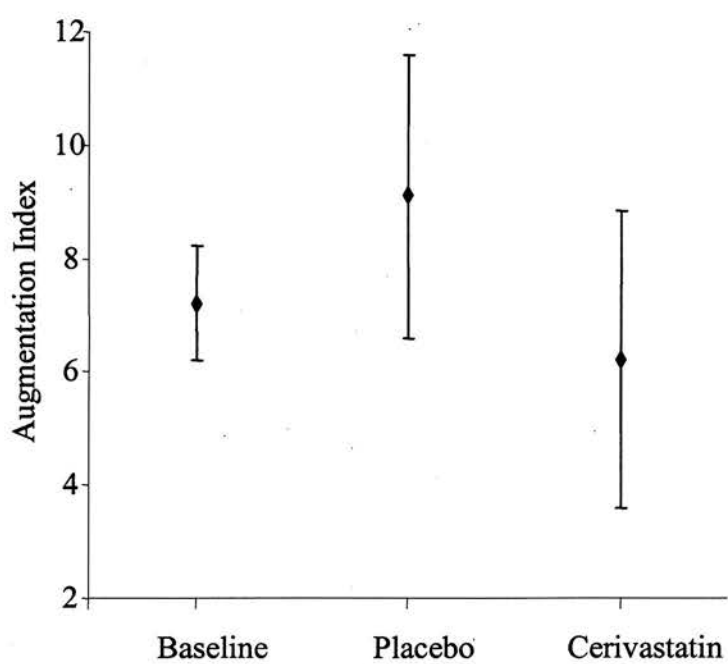
Cerivastatin therapy for 8 weeks resulted in a reduction in total plasma cholesterol compared with baseline of 27 % ( $5.4 \pm 0.4$  vs  $7.3 \pm 0.4$  mmol/L,  $p = 0.04$ ), this was achieved by a reduction in lower density lipoprotein (LDL) cholesterol (Figure 9.1). There was no effect after 8 weeks of placebo treatment ( $7.4 \pm 0.4$  vs  $7.3 \pm 0.4$  mmol/L,  $p = 0.9$ ). Compared with placebo, 8 weeks of cerivastatin therapy caused a trend towards an increase in forearm blood flow (FBF) in response to selective  $ET_A$  antagonism ( $18.0 \pm 7.2$  vs  $52.0 \pm 19.0$  %,  $p = 0.06$ ) (Figure 2). Selective  $ET_B$  receptor blockade reduced FBF with no difference between placebo and cerivastatin therapy ( $11.0 \pm 3.9$  vs  $13.0 \pm 3.6$  %,  $p = 0.9$ ) (Figure 9.2). Dual  $ET_{A/B}$  receptor blockade increased FBF with no difference between placebo and cerivastatin therapy ( $39.8 \pm 13.4$  vs  $42.4 \pm 19.0$  %,  $p = 0.7$ ). There was a trend towards a reduction in augmentation index between cerivastatin and placebo ( $6.2 \pm 2.7$  vs  $9.1 \pm 2.4$ ,  $n = 5$ ,  $p = 0.4$ ) compared with baseline ( $7.2 \pm 1.0$ ) (Figure 9.3).



**Figure 9.1** Plasma concentrations of low density lipoprotein (LDL) and high density lipoprotein (HDL) after 8 weeks treatment with placebo or cerivastatin.



**Figure 9.2** Change in forearm blood flow from baseline with intra-arterial infusion of BQ123 (open circles), BQ788 (open squares) or the combination of BQ123 and BQ788 (closed circles) following 8 weeks treatment with placebo or cerivastatin (ANOVA, AUC).



**Figure 9.3** Change in augmentation index

## 9.5 DISCUSSION

This study has demonstrated that 8 weeks of statin therapy caused a trend towards a reduction in arterial stiffness and increased vasodilatory responses to selective ET<sub>A</sub> receptor blockade. There were no differences in responses to selective ET<sub>B</sub> receptor or combined ET<sub>A/B</sub> receptor between the placebo and statin treatment periods.

Consistent with studies in healthy volunteers, selective ET<sub>A</sub> receptor blockade with BQ-123 increased forearm blood flow. However, there was a trend towards an increase in this vasodilation after treatment with cerivastatin. These preliminary results are of interest and there are several possible explanations. If the balance of effect at the ET<sub>B</sub> receptor is indeed vasodilatation then the vasodilatation seen with ET<sub>A</sub> receptor blockade may have two components, firstly the reduction in ET-1 tone mediated through the ET<sub>A</sub> receptor when blocked and secondly there will be an increase in ET<sub>B</sub> receptor mediated vasodilatation due to increased availability of ET-1 due to ET-1 displacement and reduction in the number of target receptors. The trend towards an increase in ET<sub>A</sub> mediated vasodilatation following cerivastatin would support the hypothesis that statin therapy increases NO mediated vasodilatation via the ET<sub>B</sub> receptor, given that there is no difference between groups when the ET<sub>B</sub> receptor is concomitantly blocked by combine BQ123 and BQ788 infusion. It has been previously shown that hypercholesterolaemia causes an impairment of NO mediated vasodilation and that this can be at least partially reversed by lipid lowering therapy [O'Driscoll *et al* 1997]. However, there may also be structural changes to the arterial wall which would be less likely to be reversed after 8 weeks of lipid lowering and therefore a longer treatment period may be expected to have greater effects. The potential interaction between endothelin antagonists and statin therapy is of interest and deserves further investigation.



It should be noted that this was a small, underpowered study. Recruitment for this study was difficult due to the long duration of the study and multiple study visits with 6 forearm blood flow experiments in each volunteer. Furthermore, the product cerivasatin was withdrawn from clinical use due to concerns regarding its safety and therefore the study was discontinued early.

## Chapter 10

### **Differential haemodynamic effects of selective endothelin ET<sub>A</sub> and dual ET<sub>A/B</sub> receptor blockade in patients with chronic heart failure**

**Leslie SJ**, Spratt JC, McKee SP, Strachan FE, Newby DE, Northridge DB, Denvir MA, Webb DJ. Direct comparison of selective endothelin ET<sub>A</sub> and non-selective ET<sub>A/B</sub> receptor blockade in chronic heart failure. *Heart* 2004 (in press).

## 10.1 SUMMARY

The aim of this study was to investigate the potential differential effects of selective endothelin A ( $ET_A$ ) and dual endothelin A and B ( $ET_{A/B}$ ) receptor blockade in patients with chronic heart failure. Nine patients with chronic heart failure (NYHA grade II/III) each received intravenous infusions of BQ-123 alone (selective  $ET_A$  blockade), and combined BQ-123 and BQ-788 (dual  $ET_{A/B}$  blockade) in a randomized, placebo-controlled, three-way cross-over study. Selective  $ET_A$  blockade increased cardiac output (max.  $33\pm 12\%$ ,  $p<0.001$ ), and reduced mean arterial pressure (max.  $-13\pm 4\%$ ,  $p<0.001$ ) and systemic vascular resistance (max.  $-26\pm 8\%$ ,  $p<0.001$ ), without changing heart rate ( $p=0.38$ ). Dual  $ET_{A/B}$  blockade significantly reduced the changes in all these haemodynamic variables compared with selective  $ET_A$  blockade ( $p<0.05$ ). Selective  $ET_A$  blockade reduced pulmonary artery pressure (max.  $25\pm 7\%$ ,  $p=0.01$ ) and pulmonary vascular resistance (max.  $72\pm 39\%$ ,  $p<0.001$ ). However, there was no difference between these effects and those seen with dual  $ET_{A/B}$  blockade. Unlike selective  $ET_A$  blockade, dual  $ET_{A/B}$  blockade increased plasma endothelin-1 concentrations (by  $47\pm 4\%$  low dose and  $61\pm 8\%$  high dose,  $p<0.05$ ). Whilst there appeared to be similar reductions in pulmonary pressures with selective  $ET_A$  and dual  $ET_{A/B}$  blockade, selective  $ET_A$  blockade causes greater systemic vasodilatation and did not affect endothelin-1 clearance. We conclude that there are significant haemodynamic differences between selective  $ET_A$  and dual  $ET_{A/B}$  blockade, which may determine responses in individual patients.

## 10.2 BACKGROUND

Endothelin-1 (ET-1) is a potent endogenous vasoconstrictor in man and contributes to the maintenance of basal vascular tone [Haynes *et al* 1994b] and blood pressure [Haynes *et al* 1996] in healthy people and patients with hypertension [Krum *et al* 1998]. It acts through two receptor subtypes; the endothelin A (ET<sub>A</sub>) and endothelin B (ET<sub>B</sub>) receptors. While both receptors are expressed on vascular smooth muscle cells and mediate vasoconstriction, only the ET<sub>B</sub> receptor is located on the endothelium where it produces a prostanoid and nitric oxide mediated vasodilation. Thus, ET<sub>B</sub> receptor mediated effects are complex, and include vasoconstriction, endothelium dependent vasodilation and a role in the clearance of ET-1. In healthy subjects, in contrast to the vasodilator and vasodepressor effects of ET<sub>A</sub> receptor blockade [Verhaar *et al* 1998, Spratt *et al* 2001], systemic ET<sub>B</sub> receptor blockade has vasoconstrictor and pressor effects [Strachan *et al* 1999], suggesting that the vascular balance of basal ET<sub>B</sub> receptor activation favors vasodilation.

Chronic heart failure is associated with neurohumoral activation as a consequence of reductions in cardiac reserve, systemic blood pressure and renal perfusion. This leads to peripheral vasoconstriction, increased systemic vascular resistance, and sodium and water retention, that together increase cardiac work and further compromise cardiac performance. Many regulatory mechanisms are involved in this maladaptive response, including the renin-angiotensin, sympathetic nervous and vasopressin systems. The endothelin system also appears to contribute to the pathophysiology of heart failure, which is associated with increased plasma ET-1 concentrations [Hiroe *et al* 1991, McMurray *et al* 1992] that correlate with changes in haemodynamics

[Cody *et al* 1992, Wei *et al* 1994], reduced exercise capacity [Krum *et al* 1995] and a poor prognosis [Pousset *et al* 1997].

In patients with chronic heart failure, systemic administration of both selective ET<sub>A</sub> [Cowburn *et al* 1998a, Givertz *et al* 2000, Speiker *et al* 2000] and dual ET<sub>A/B</sub> blockade [Kiowski *et al* 1995, Packer *et al* 1998, Sutsch *et al* 1998, Schalcher *et al* 2001] reduces systemic vascular resistance and increases cardiac output. In patients with acute decompensated heart failure systemic administration of dual ET<sub>A/B</sub> blockade has been shown to have beneficial effects [Torre-Amione *et al* 2003]. However, systemic ET<sub>B</sub> blockade increases systemic vascular resistance and has potentially detrimental effects in patients with chronic heart failure [Cowburn *et al* 2001]. Therefore, the question arises as to whether selective ET<sub>A</sub> or dual ET<sub>A/B</sub> blockade is likely to be the favored therapeutic approach in patients with heart failure. However, to date, there have been no direct studies comparing these two approaches.

### **10.3 AIMS**

The aims of this placebo-controlled study, in patients with stable chronic heart failure, were to compare in a head-to-head manner the effects of selective ET<sub>A</sub> blockade with dual ET<sub>A/B</sub> blockade on systemic and pulmonary hemodynamics.

## **10.4 METHODS**

### **10.4.1 Patient selection**

Nine patients with chronic heart failure (New York Heart Association Grade II/III) due to left ventricular dysfunction were recruited if they had an ejection fraction  $\leq$  35% (by echocardiography using the biplanar Simpson's rule) and had been stable on treatment, including angiotensin converting enzyme inhibitor or angiotensin receptor antagonist therapy, for at least 3 months. Patients were all in sinus rhythm and no patient had a pacemaker or implantable cardiac defibrillator. Patients were excluded if they had insulin dependent diabetes mellitus, abnormal liver function, renal impairment (creatinine  $> 200 \mu\text{mol/L}$  for men;  $> 180 \mu\text{mol/L}$  for women) or a systolic blood pressure  $> 190$  or  $< 90$  mmHg or, within 3 months, had undergone coronary artery bypass graft surgery, percutaneous coronary intervention or had an acute coronary syndrome, myocardial infarction or cerebrovascular accident

The study was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. Written informed consent was obtained from each subject before entry into the study.

### **10.4.2 Measurements**

Blood pressure and heart rate were measured non-invasively using a Dynamap<sup>TM</sup> compact TS (Critikon LLC, Ascot, UK). Cardiac output, mean pulmonary artery pressure, pulmonary artery wedge pressure and central venous pressure were measured continuously using a single multi-lumen thermodilution cardiac output pulmonary artery catheter (Swan-Ganz CCOmbo - CCO/SVO<sub>2</sub>; Edwards

Lifesciences LLC, Irvine California, USA). Cardiac output was calculated automatically (Vigilence, Edwards Critical Care, Baxter's Healthcare Corporation, Irvine, CA, USA) and, at each time point, the cardiac output was taken as the mean of three measurements.

#### **10.4.3 Protocol**

All patients attended fasted at 7.30 am on three occasions at least one week apart. Patients were asked to omit their regular medications on the morning of the study. The studies were conducted in a quiet, draught free room maintained at a constant temperature (22-24 °C). A pulmonary artery catheter was inserted, via a 9F femoral venous sheath, into the right pulmonary artery and was flushed with 0.9% heparinized saline. Before starting drug administration, patients underwent an equilibration period of > 90 min until blood pressure, heart rate and cardiac output were stable, with 3 consecutive measurements within 10%. Study drugs were administered by 15 min infusion in 2 incremental doses 60 min apart. Although for safety reasons an attending physician was not blinded to the treatment, all variables were recorded in a double blind manner by separate investigators.

#### **10.4.4 Drug administration**

A venous cannula for drug administration was inserted under local anesthesia. Pharmaceutical grade BQ-123 and BQ-788 (Clinalfa AG, Läufelfingen, Switzerland) were dissolved in 0.9% saline (Baxter Healthcare Ltd, Thetford, UK). On each study day, patients received a low dose infusion at  $t = 0$  for 15 min followed by a high dose infusion at  $t = 60$  min for 15 min. On different study days and in random order,

patients received saline placebo, BQ-123 (low dose, 1.5  $\mu\text{mol}$ ; high dose, 15  $\mu\text{mol}$ ) alone or the co-infusion of BQ-123 (low dose, 1.5  $\mu\text{mol}$ ; high dose, 15  $\mu\text{mol}$ ) and BQ-788 (low dose, 0.45  $\mu\text{mol}$ ; high dose, 4.5  $\mu\text{mol}$ ). These doses were selected following studies in healthy volunteers using BQ-123 and BQ-788. This dose of BQ-123 was sufficient to reduce systemic vascular resistance and block the effects of local infusion of ET-1 into the forearm [Spratt *et al* 2001]. This dose of BQ-788 was sufficient to reduce systemic vascular resistance [Strachan *et al* 1999]. The detailed rationale for these doses has been discussed elsewhere [Goddard *et al* 2002].

#### **10.4.5 Blood sampling and plasma assays**

Venous blood for ET-1 and big-ET-1 (the 38 amino-acid precursor of ET-1) assay was taken from the femoral vein. Blood was collected into 0.16% EDTA (Sarstedt, Aktiengesell Schaft & Co, Germany) and immediately separated by centrifugation (2500 *g* for 20 min at 4°C) and stored at -80 °C until analysis. Following extraction using Bond Elut® columns (Varian, Harbor City, CA, USA), ET-1 (Peninsula Laboratories Europe Ltd, St Helens, UK) and big ET-1 (Peninsula Laboratories Europe Ltd) concentrations were determined by radioimmunoassay as previously described [Newby *et al* 1998]. The intra-assay coefficients of variability were 7.0 and 7.2% respectively and the inter-assay coefficients of variability were 9.0 and 9.3% respectively. These assays were performed by Mr Neil Johnston.

#### **10.4.6 Data analysis and statistics**

Data are expressed as change from baseline  $\pm$  SEM or mean area under the curve (AUC)  $\pm$  SEM unless otherwise specified. Data were examined by analysis of



variance (ANOVA) with repeated measures over time and Student's *t*-test with correction for multiple measures where appropriate (Excel v5.0, Microsoft). Statistical significance was taken at the 5% level.

## **10.5 RESULTS**

Baseline patient characteristics and medications are shown in Table 10.1. There were no adverse events and the study was well tolerated by all patients. There were no significant differences in baseline hemodynamic variables between study visits (Table 10.2). Placebo administration caused no significant changes in hemodynamic variables throughout the course of the study (ANOVA  $p>0.9$ ).

### **10.5.1 Cardiac output and heart rate**

In comparison to placebo, BQ-123 alone (AUC:  $p<0.001$ ), but not BQ-123/788 (AUC:  $p=0.08$ ), increased cardiac output with a maximum increase of  $33\pm12\%$  at 75 min. Infusion of BQ-123 alone increased cardiac output compared with BQ-123/788 (AUC:  $p<0.001$ ) (Figures 10.1c & 10.3). There was no significant change in heart rate with either BQ123 alone (AUC:  $p=0.38$ ) or BQ-123/788 (AUC:  $p=0.39$ ) (Figures 10.1a & 10.3).

### **10.5.2 Left ventricular filling pressure and systemic hemodynamics**

In comparison to placebo, BQ-123 alone (AUC:  $p=0.01$ ) and BQ-123/788 (AUC:  $p<0.01$ ), reduced pulmonary artery wedge pressure by a maximum of  $19\pm7\%$  at 150 min and  $26\pm7\%$  at 105 min respectively (Figures 10.2c & 10.3). There was no difference between the magnitude of reduction in pulmonary artery wedge pressure between BQ-123 alone and BQ-123/788 (AUC:  $p=0.47$ ). BQ-123 alone (AUC:  $p<0.001$ ) and BQ-123/788 (AUC:  $p<0.05$ ) reduced mean arterial pressure by a maximum of  $14\pm5\%$  and  $12\pm4\%$  respectively at 150 min. BQ-123 alone reduced

mean arterial pressure to a greater degree than BQ-123/788 (AUC:  $p<0.05$ ) (Figures 10.1b & 10.3).

BQ-123 alone (AUC:  $p<0.001$ ) and BQ-123/788 (AUC:  $p<0.05$ ) reduced systemic vascular resistance by a maximum of  $26\pm 8\%$  and  $16\pm 5\%$  respectively at 75 min in comparison to placebo. BQ-123 alone reduced systemic vascular resistance to a greater degree than BQ-123/788 (AUC:  $p<0.05$ ) (Figures 10.1d & 10.3).

### **10.5.3 Right ventricular filling pressure and pulmonary hemodynamics**

In comparison to placebo, neither BQ-123 alone (AUC:  $p=0.17$ ) or BQ-123/788 (AUC:  $p=0.69$ ) changed central venous pressure (Figure 10.2a & 10.3). BQ-123 alone (AUC:  $p=0.01$ ) and BQ-123/788 (AUC:  $p=0.02$ ) reduced mean pulmonary arterial pressure by a maximum of  $25\pm 7\%$  and  $26\pm 6\%$  respectively at 90 min. There was no significant difference between these responses (AUC:  $p=0.98$ ). (Figures 10.2b and 10.3).

In comparison to placebo, both BQ-123 alone and BQ-123/788 (AUC: both  $p<0.001$ ) reduced pulmonary vascular resistance by a maximum of  $72\pm 39\%$  and  $40\pm 16\%$  respectively at 75 min. There was no significant difference between these responses (AUC:  $p=0.49$ ) (Figures 10.2d & 10.3).

### **10.5.4 Plasma ET-1 and big ET-1**

There was no change in plasma concentrations of big ET-1 with placebo, BQ-123 alone or BQ-123/788. There was no significant change in plasma ET-1

concentrations with placebo or BQ-123 alone, whereas BQ-123/788 caused an increase in plasma ET-1 concentrations (47% low dose and 61% high dose, both  $p < 0.05$ )(Figure 10.4).

Patient	Age	BMI	BP (mmHg)	Heart Rate (mmHg)	MPAP (mmHg)	PAWP (mmHg)	Cause of Heart Failure	NYHA	Drugs
1	75	26	186/79	52	20	11	Ischemic	III	ASA75, En20, Bu1, Sim20
2	63	23	120/74	64	13	6	Idiopathic	II	ASA75, En20
3	60	27	122/58	62	17	12	Ischemic	III	Val80, Frus40
4	43	30	98/64	77	12	8	Ischemic	II	ASA75, Lis10, Frus40
5	56	29	139/88	96	11	6	Ischemic	III	ASA75, Lis20, Frus40, Car12.5, Dig250, Sim20,
6	61	26	136/77	66	16	12	Ischemic	III	ASA75, Lis10, Frus40, Spir25, Dig125, Sim20
7	52	36	184/106	94	25	23	Ischemic	III	Lis15, Frus20, Aten100
8	74	33	143/75	58	14	10	Ischemic	III	ASA75, Lis10, Frus40, Bis10, Sim10
9	67	21	154/78	65	14	10	Ischemic	II	ASA75, Lis10, Aten50, Pra20
Average	61	28	142/78	70	16	11			
SEM	3	1	9/4	5	1	2			

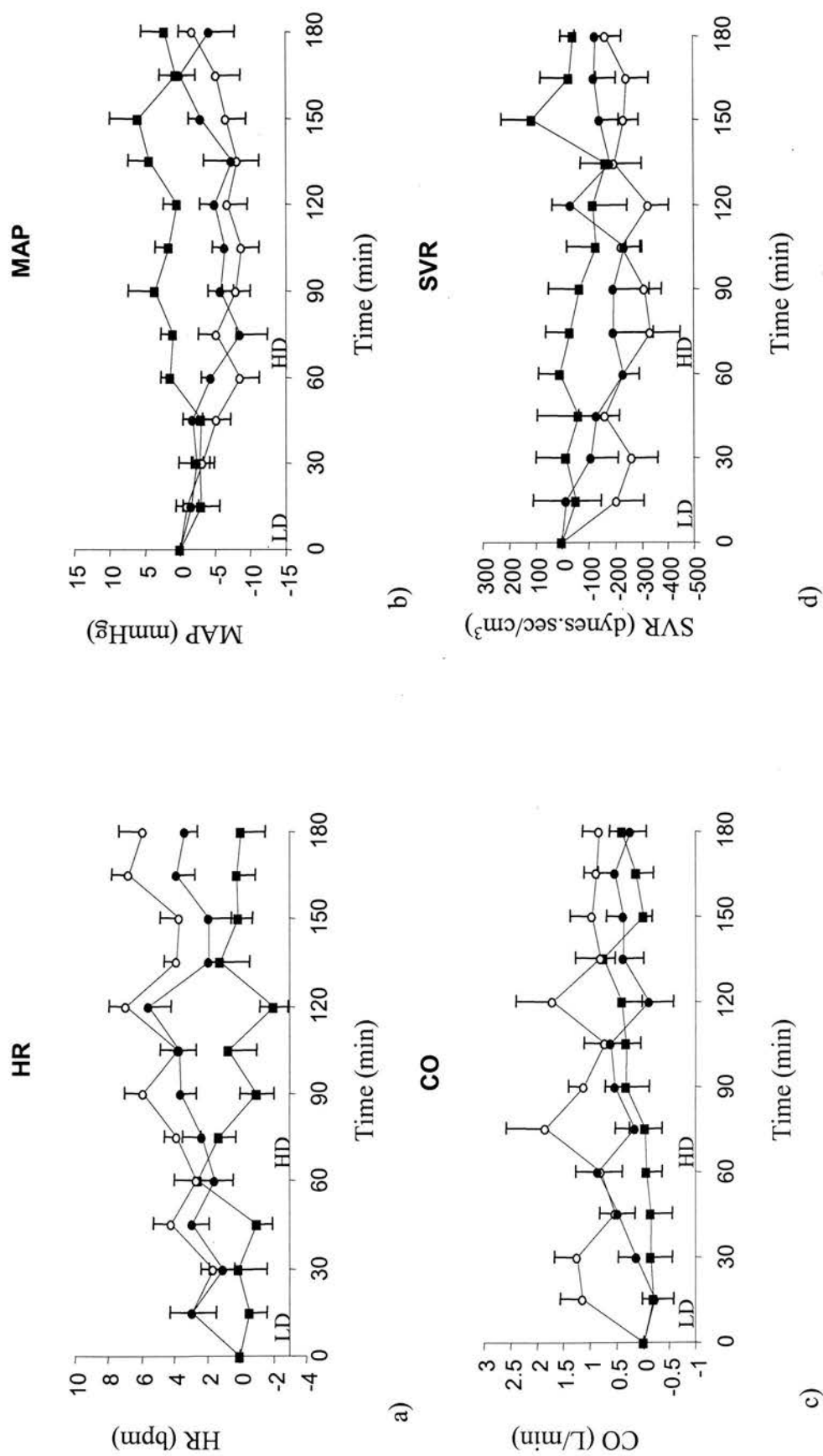
Table 10.1 Patient Characteristics and Medication

Body mass index (BMI), Blood pressure (BP), Aspirin (ASA), Atenolol (Aten), Bisoprolol (Bis), Bumetanide (Bu), Carvedilol (Car), Digoxin (Dig), Enalapril (En), Frusemide/Amiloride (Frum), Frusemide (Frus), Lisinopril (Lis), Pravastatin (Pra), Simvastatin (Sim), Spironolactone (Spir) and Valsartan (Val) (doses in mg except Digoxin (µg)).

Parameter	Placebo (n=9)	BQ123 (n=9)	BQ123 + BQ788 (n=9)
HR (bpm)	64 ± 5	62 ± 4	63 ± 5
MAP (mmHg)	91 ± 7	87 ± 6	90 ± 7
SBP (mmHg)	106 ± 8	104 ± 9	106 ± 9
DBP (mmHg)	69 ± 4	68 ± 6	71 ± 4
CVP (mmHg)	5 ± 1	5 ± 1	3 ± 2
CO (L/min)	5.0 ± 0.3	5.5 ± 0.4	5.3 ± 0.3
SVR (dynes.sec/cm <sup>-5</sup> )	1462 ± 150	1266 ± 138	1344 ± 138
MPAP (mmHg)	16 ± 1	16 ± 1	17 ± 2
PAWP (mmHg)	11 ± 2	11 ± 1	12 ± 3
PVR (dynes.sec/cm <sup>-5</sup> )	83 ± 12	91 ± 14	78 ± 18

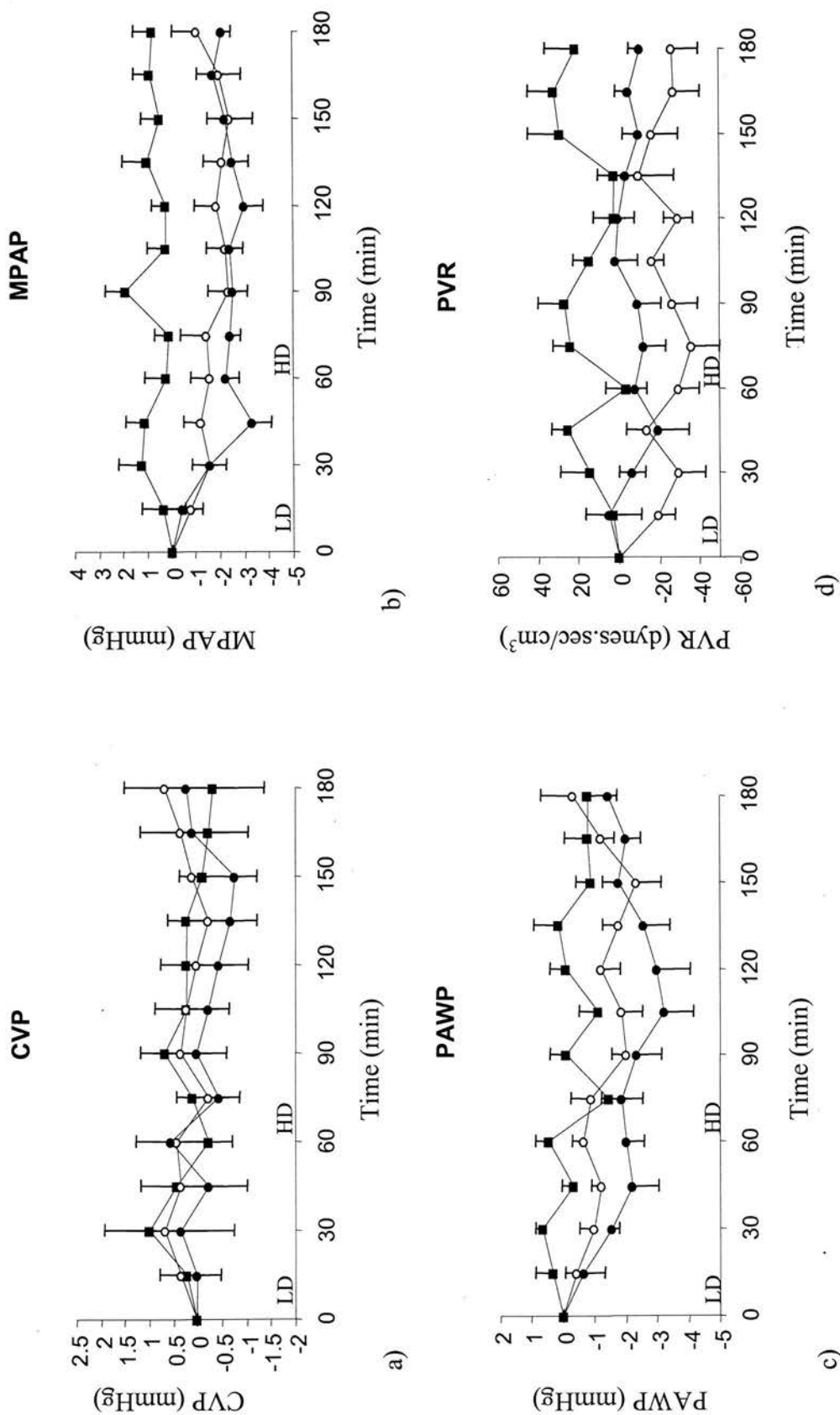
**Table 10.2** Baseline parameters

Heart rate (HR), mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), central venous pressure (CVP), cardiac output (CO), systemic vascular resistance (SVR), mean pulmonary artery pressure (MPAP), pulmonary artery wedge pressure (PAWP) and pulmonary vascular resistance (PVR).



**Figure 10.1**

Effect of selective  $ET_A$  blockade (open circles), dual  $ET_A/B$  blockade (closed circles) and placebo (closed squares) on heart rate (HR), mean arterial pressure (MAP), cardiac output (CO) and systemic vascular resistance (SVR), at low dose (LD) and high dose (HD).

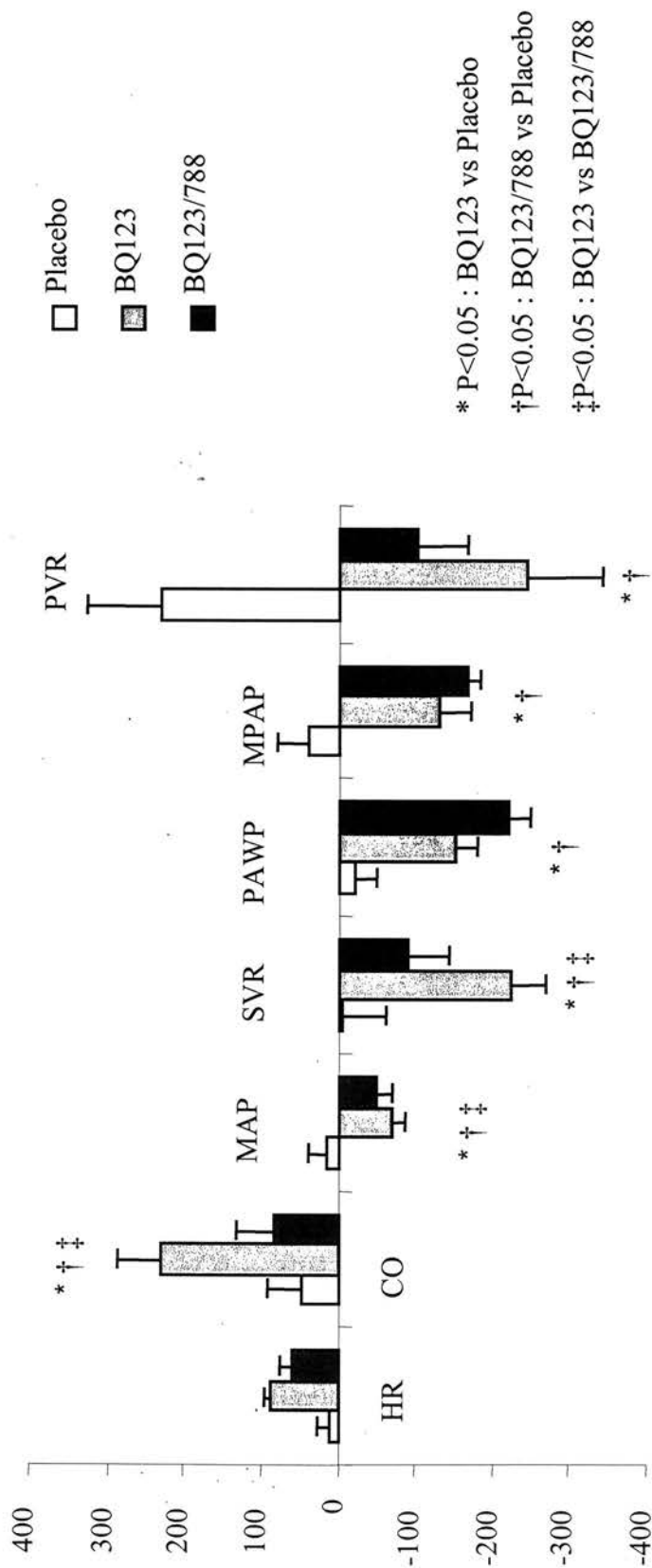


**Figure 10.2**

Effect of selective  $ET_A$  blockade (open circles), dual  $ET_{A/B}$  blockade (closed circles) and placebo (closed squares) on central venous pressure (CVP), mean pulmonary arterial pressure (MPAP), pulmonary artery wedge pressure (PAWP) and pulmonary vascular resistance (PVR), at low dose (LD) and high dose (HD).



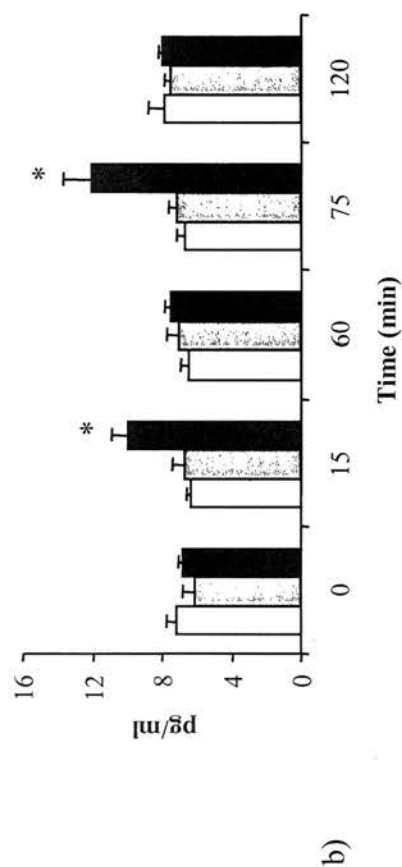
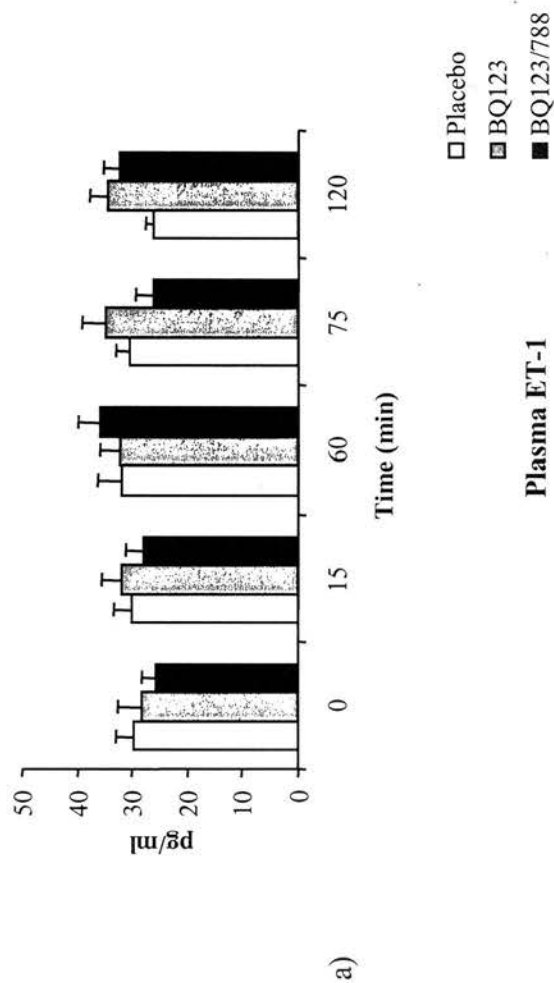
# AUC change from baseline



**Figure 10.3**

Comparison of the hemodynamic effects of placebo (white), selective ETA blockade (gray) and dual ETA/B blockade (black) on heart rate (HR), cardiac output (CO), mean arterial pressure (MAP), systemic vascular resistance (SVR), pulmonary arterial wedge pressure (PAWP), mean pulmonary artery pressure (MPAP) and pulmonary vascular resistance (PVR) (ANOVA, of AUC).

# Plasma big ET-1



**Figure 10.4**

Effects of placebo (white), selective ET<sub>A</sub> blockade (grey) and combined ET<sub>A/B</sub> blockade (black) on plasma concentration of big ET-1 (a) and ET-1 (b). (\*  $p < 0.05$  vs baseline, one tailed, paired Student's  $t$ -test)

## 10.6 DISCUSSION

In this randomised placebo-controlled crossover study, we have shown that there are significant haemodynamic differences between the responses to selective ET<sub>A</sub> and dual ET<sub>A/B</sub> receptor blockade in patients with chronic heart failure. Both selective ET<sub>A</sub> and dual ET<sub>A/B</sub> receptor blockade increased cardiac output and reduced mean arterial pressure and systemic vascular resistance. However, selective ET<sub>A</sub> receptor blockade caused a greater increase in cardiac output and reduction in systemic vascular resistance than dual ET<sub>A/B</sub> receptor blockade. In contrast, selective ET<sub>A</sub> and dual ET<sub>A/B</sub> blockade caused similar reductions in both pulmonary artery pressure and pulmonary vascular resistance. There was a greater reduction in pulmonary artery pressure with dual blockade compared with selective ET<sub>A</sub> blockade after low dose infusion although this difference was not apparent after high dose infusion.

This study directly compared systemic selective ET<sub>A</sub> and dual ET<sub>A/B</sub> blockade in patients with heart failure. Our findings are consistent with other short-term studies which have demonstrated that both selective ET<sub>A</sub> [Speiker *et al* 2000] and dual ET<sub>A/B</sub> [Sutsch *et al* 1998, Schalcher *et al* 2001, Torre-Amione *et al* 2003] blockade increase cardiac output and reduce systemic vascular resistance, importantly with no change in heart rate. Both selective ET<sub>A</sub> [Givertz *et al* 2000, Speiker *et al* 2000] and dual ET<sub>A/B</sub> [Sutsch *et al* 1998, Schalcher *et al* 2001, Torre-Amione *et al* 2003] blockade also reduce pulmonary vascular resistance and pulmonary artery wedge pressure. Comparing magnitude of response between different endothelin antagonists in different patient populations is difficult but we have demonstrated in this head-to-

head study that selective endothelin blockade had greater effects on the systemic vasculature than dual endothelin antagonism.

There is increasing evidence that the  $ET_B$  receptor has a role in the clearance of plasma ET-1. Plasma ET-1 concentration increases following systemic selective  $ET_B$  blockade in healthy subjects [Strachan *et al* 1999] and following dual  $ET_{A/B}$  blockade in healthy subjects [Plumpton *et al* 1996, Krum *et al* 1998] and patients with chronic heart failure [Kioski *et al* 1995, Plumpton *et al* 1996, Krum *et al* 1998, Sutsch *et al* 1998]. However, the effects of systemic  $ET_A$  blockade alone are less consistent with little, if any, increase in plasma ET-1 concentrations in most studies, [Verhaar *et al* 1998, Cowburn *et al* 1998a, Givertz *et al* 2000] although one study did report an increase at higher degrees of  $ET_A$  blockade [Speiker *et al* 2000]. These results are confirmed in our study, in which selective  $ET_A$  receptor blockade had no effect on plasma ET-1 concentrations whereas they were increased by dual blockade. Because there was no change in plasma big ET-1 concentration, elevation of plasma ET-1 is likely to reflect interference with its clearance rather than an increase in its synthesis and release. Thus, selective  $ET_A$  blockade has a theoretical benefit of leaving the endothelin clearance receptor ( $ET_B$ ) functional. Nevertheless, if the  $ET_A$  receptor is also effectively blocked, during  $ET_B$  receptor blockade, then the high circulating concentrations of ET-1 may not be of clinical importance.

Although selective  $ET_A$  receptor blockade had greater effects on systemic vascular resistance, there may be clinical situations in which blockade of the  $ET_B$  receptor is desirable. There is a higher density of  $ET_B$  receptors in the pulmonary vasculature,

and these may be upregulated in pulmonary arterial hypertension [McCulloch *et al* 1995]. Also, endothelin-1 release across the pulmonary vascular bed correlates strongly with the pulmonary vascular resistance in chronic heart failure [Tsutamoto *et al* 1994]. Raised pulmonary artery pressure is an independent risk factor in chronic heart failure and responds poorly to conventional therapies. Here, we demonstrated that both selective ET<sub>A</sub> and dual ET<sub>A/B</sub> receptor blockade reduced pulmonary artery pressures.

Endothelin antagonism might, therefore, be of benefit in patients with heart failure and raised pulmonary artery pressures. Indeed, the dual antagonist, bosentan, has recently been approved for use in the treatment of primary pulmonary arterial hypertension based on its effectiveness in this situation. The long-term clinical effects of endothelin receptor blockade in patients with pulmonary hypertension secondary to chronic heart failure are unknown but it is tempting to speculate that it may also be more effective in this setting. We have failed to demonstrate convincingly whether there are true haemodynamic differences between selective ET<sub>A</sub> and dual ET<sub>A/B</sub> receptor antagonism in the pulmonary circulation. However, none of the patients in the present study had significant pulmonary hypertension. We believe that the role of endothelin antagonism now warrants further careful assessment in a much larger trial of patients with both heart failure and a significant degree of pulmonary hypertension.

Many studies use agents that, while termed 'selective' or 'dual' inhibitors of ET<sub>A</sub> and ET<sub>B</sub> receptors, have a range of receptor selectivities, mostly inhibiting the ET<sub>A</sub>

receptor at much lower concentrations than at the ET<sub>B</sub> receptor [Goddard *et al* 2002]. In this study we have used two receptor antagonists, BQ-123 and BQ-788, given separately, and with selectivity for the ET<sub>A</sub> and ET<sub>B</sub> receptor respectively. Therefore, it is important to recognize that we have examined mechanistically the influence of major blockade of the ET<sub>B</sub> receptor on responses to full ET<sub>A</sub> blockade. This may not exactly represent the clinical situation that exists with dual antagonists, such as bosentan, which are relatively selective for the ET<sub>A</sub> receptor (ET<sub>A</sub> : ET<sub>B</sub> selectivity > 10). The doses of BQ-123 given here have been shown to produce maximum systemic haemodynamic effects, and block responses to forearm artery infusion of ET-1, but not to increase plasma ET-1 concentrations. Given that BQ-123 caused greater systemic vasodilatation than the combination with BQ-788, the overall hemodynamic effect of ET<sub>B</sub> blockade in patients with heart failure is likely to be vasoconstriction, a finding consistent with other work [Love *et al* 2000, Cowburn *et al* 2001].

This was an acute haemodynamic study and we have not assessed whether these effects are sustained in the long-term. Nevertheless, previous haemodynamic studies indicate that the acute effects of both selective ET<sub>A</sub> [Luscher *et al* 2002] and dual [Sutsch *et al* 1997] endothelin receptor blockade are maintained or enhanced over several weeks and therefore likely to be sustained. The clinical impact of these haemodynamic changes is, of course, uncertain and can only be clarified in the context of large-scale clinical outcome studies. We have demonstrated that selective ET<sub>A</sub> blockade causes more marked systemic vasodilatation than dual ET<sub>A/B</sub> receptor blockade in NYHA grade II/III patients with heart failure. To date, there have been

only two, as yet unpublished, large scale, randomized controlled trials of endothelin receptor blockade in patients with heart failure (NYHA grade III/IV), both of which have demonstrated no major clinical benefit of either bosentan ( $ET_A: ET_B$  selectivity  $\sim 10$ ) [Coletta *et al* 2002] or darusentan ( $ET_A : ET_B$  selectivity  $> 500$ ) [Luscher *et al* 2002].

It should be noted that some of these patients would be considered now to be 'undertreated'. In particular the poor use of beta-blockers and the persistent hypertension, may affect the applicability of these results to more contemporary cohorts of heart failure patients. Furthermore, these patients had mild to moderate rather than severe heart failure which may limit the applicability of these results to all heart failure populations. In addition, aspirin use may confound results. Aspirin by inhibiting cyclo-oxygenase will reduce PGE<sub>2</sub> and PGI<sub>2</sub> reducing the potential vasodilator effects of  $ET_B$  receptor blockade. Nevertheless, the majority of patients with heart failure will also have coronary artery disease and aspirin is integral to standard treatment in these patients.

## Conclusions

In this study both selective  $ET_A$  and dual  $ET_{A/B}$  blockade caused acute systemic and pulmonary haemodynamic changes in patients with heart failure. However, important differences exist and selective  $ET_A$  blockade causes greater systemic haemodynamic effects than dual  $ET_{A/B}$  blockade. There is now a need for further trials to determine whether selective or dual endothelin antagonism has greater potential efficacy in the treatment of patients with heart failure.

## **Chapter 11**

### **Summary and Discussion**



## 11.1 INTRODUCTION

This thesis represents a study of the role of the endothelin system in health and disease. In addition to studies of whole body haemodynamics, I have attempted to assess its role in specific vascular beds (i.e. skin, pulmonary) and in specific tissues (myocardium). Using a range of techniques, some of which I have developed and validated, I have demonstrated the diverse and complex role of the endothelin system in health and disease. The key findings and their implications for therapeutic option are discussed in this final chapter.

Since its discovery [Yanagisawa *et al* 1988], the endothelin system has been extensively investigated and although much is known, important questions remain unanswered, not least being whether pharmacological manipulation of the endothelin system in disease states will prove to be beneficial to patients.

## 11.2 Techniques to investigate the cardiovascular system

In order to investigate vasosactive compounds in the preclinical setting, especially when the cardiovascular effects are unknown, various techniques are required. The relative merits of *in vitro* versus *in vivo* and local versus systemic techniques in the investigation of the cardiovascular system were discussed in Chapter 2. This thesis has used and evaluated several such techniques including isolated myocardial trabeculae force experiments (Chapter 8), skin blood flowmetry (Chapter 6), forearm plethysmography (Chapter 9), systemic haemodynamic techniques and neurohormonal assays (Chapter 10)

Skin blood flowmetry, forearm plethysmography and systemic haemodynamic techniques have been validated in Chapters 3, 4 and 5 respectively. In addition, a new assay for the measurement of ET-1<sub>[1-31]</sub> was assessed in Chapter 7.

### **11.2.1 Skin blood flowmetry**

The data in Chapter 3 demonstrated that skin blood flowmetry coupled with intra-dermal injection of vasoactive compounds offers a reasonably reproducible technique when large vasoactive molecules, which cannot be delivered by other techniques such as iontophoresis. In addition, the skin microcirculation offers an advantage over systemic studies in that small doses of compound can be used and multiple sites employed simultaneously, making this a relatively safe and well tolerated technique for *in vivo* human studies. This technique was subsequently employed in Chapter 6 to investigate the vasoactive effects of ET-1, big ET-1, BQ123 (selective ET<sub>A</sub> receptor antagonist), BQ788 (selective ET<sub>B</sub> receptor antagonist), phosphoramidon (ECE/NEP inhibitor) and the recently discovered ET-1<sub>[1-31]</sub>.

### **11.2.2 Forearm plethysmography**

There continue to be advances in the design of devices for measuring forearm blood flow. The advantage of forearm plethysmography, especially when coupled with intra-arterial brachial infusion is that resistance vessels can be studied, in isolation, *in vivo*. In Chapter 4, a newly developed device, Filtrass 2001, was validated against the established Hokanson HEC4. Each device was compared with itself and the other to give a measure of repeatability and to assess systematic bias. The devices were assessed over a range of blood flows. Small (~30%) increases and decreases in blood

flow were achieved using Stroop's coloured word test and lower body negative pressure respectively. Greater increases in blood flow (up to 800%) were achieved by graded arterial occlusion resulting in post occlusive hyperaemia. Although the Filtrass 2001 offered some practical advantages over the HEC4 such as automated data analysis and a more compact unit of smaller size, there were no important advantages in terms of repeatability and therefore for subsequent experiments in Chapter 9, the established HEC4 was used. The use of blood flow in the dominant forearm as a contemporaneous control for the effects on blood flow in the non-dominant arm and of intra-arterial drug infusion is widely used in the technique of forearm plethysmography [Benjamin *et al* 1995, Wilkinson & Webb 2001]. This is supported by this study with only ~ 1% difference in percentage change in FBF from baseline between left and right arms when measured by the HEC4.

### **11.2.3 Systemic haemodynamic monitoring**

There is no 'perfect' method for measuring cardiac output although the technique of thermodilution is considered the 'gold standard'. However, due to the invasive nature of pulmonary artery catheterisation there is considerable interest in the non-invasive technique of thoracic bioimpedance. Thoracic bioimpedance has the advantage that it is relatively inexpensive and easy to use. However, its accuracy has been questioned. In Chapter 10, invasive haemodynamic monitoring using a pulmonary artery catheter and thermodilution was employed to monitor systemic effects of endothelin antagonism in patients with CHF. The opportunity was taken to assess the non-invasive technique of thoracic bioimpedance against invasive thermodilution measurements. Thoracic bioimpedance has been previously assessed in unstable

patients in the clinical setting and in patients with stable patients undergoing routine investigation. In Chapter 5 the use of thoracic bioimpedance during an acute pharmacological intervention designed to alter systemic haemodynamics is reported. This study compared the techniques of thoracic bioimpedance against thermodilution in stable patients with chronic heart failure undergoing an acute haemodynamic intervention study (Chapter 10). A previous meta-analysis of thoracic bioimpedance studies found a correlation between thoracic bioimpedance and reference techniques ( $r = 0.66$ ) [Fuller 1992]. In keeping with these studies we have found a reasonable correlation between thoracic bioimpedance and a thermodilution technique ( $r = 0.76$ ). However, while there is a positive correlation between the techniques, there is a poor overall level of agreement. The method of thoracic bioimpedance systematically underestimated cardiac output compared with thermodilution, and this difference appeared to be greater with higher cardiac output. Several studies have supported the use of thoracic bioimpedance when measuring intra-patient trends in cardiac output [Northridge *et al* 1990, Thomas *et al* 1992, Kööbi *et al* 1997, Salandin *et al* 1988, Belardinelli *et al* 1996, Barin *et al* 2000]. However, the results in Chapter 5 do not support this finding with poor agreement between values when expressed as percentage change from baseline despite reasonable repeatability of baseline measurements.

We observed an unacceptably low level of agreement both for absolute and percentage changes in cardiac output. Previous studies have assessed thoracic bioimpedance in critically ill patients or patients after mechanical ventilation or undergoing cardiac surgery. Pulmonary oedema and mechanical ventilation can

affect thoracic bioimpedance and many previously studied patients may have been unsuitable for measurement of cardiac output by thoracic bioimpedance. The patients in Chapter 5 had stable chronic heart failure, were in sinus rhythm and in the controlled setting of an acute haemodynamic intervention study. It was expected that these optimal conditions would provide the best opportunity for thoracic bioimpedance to be comparable with the gold standard of thermodilution, given that patients were stable, self-ventilating and had no pulmonary oedema. However, the poor agreement found suggests that thoracic bioimpedance is not comparable to thermodilution even in patients with stable chronic heart failure.

### **11.3 The endothelin system in healthy man**

The vascular effects of endothelin are mediated through 2 receptors. The ET<sub>A</sub> and ET<sub>B</sub> receptors are both found on the vascular smooth muscle cell mediating vasoconstriction. However, the ET<sub>B</sub> receptor is also found on the endothelial cell where it causes a nitric oxide and prostanoid mediated vasodilatation. Thus, the net effect of endothelin agonism will be a balance between these vasoconstricting and vasodilating effects.

In healthy individuals, under resting conditions, endothelin plays an important role in the maintenance of normal vascular tone [Haynes *et al* 1994b], mostly via the ET<sub>A</sub> receptor as blockade of the ET<sub>A</sub> receptor results in vasodilation in forearm blood flow studies and in systemic studies a reduction in systemic vascular resistance and blood pressure. Furthermore, clinical studies have demonstrated that selective ET<sub>A</sub> and dual ET<sub>A/B</sub> receptor blockade results in sustained reductions in blood pressure in

both healthy patients and patients with essential hypertension. However, to date there have been no head to head clinical studies comparing selective with dual blockade. In addition, there are concerns regarding elevations in liver enzymes and currently there are no endothelin antagonists licensed for hypertension. Due to the large number of currently available antihypertensive agents, many with good tolerability and evidence of mortality benefits, it could be argued that it is difficult to envisage a role for endothelin antagonists in the treatment of systemic hypertension. An alternative strategy might be the blockade of endothelin production. To date, there have not been any large scale clinical trials involving selective endothelin converting enzyme inhibitors although combined ACE, NEP and ECE inhibitors are under investigation as alternative vasodilator agents. Given the disappointing results of trials involving endothelin receptor antagonists, this alternative strategy may be of interest.

### **11.3.1 Endothelins in the healthy human skin microcirculation**

In Chapter 6, in the human skin microcirculation, I have confirmed that ET-1<sub>[1-21]</sub> is a potent vasoconstrictor and shown for the first time that ET-1<sub>[1-31]</sub> and big ET-1<sub>[1-38]</sub> also cause skin vasoconstriction *in vivo*. These results suggest that there is ECE activity in the skin as demonstrated by the vasoconstriction following intradermal administration of big ET-1<sub>[1-38]</sub> and vasodilatation with ECE inhibition. In addition, we have confirmed that selective blockade of the ET<sub>A</sub> receptor caused skin microvasculatory vasodilatation [Wenzel *et al* 1998] and that selective blockade of the ET<sub>B</sub> receptor also results in vasodilatation. In contrast to observations in resistance vessels, this suggests that ET<sub>B</sub> receptors in the skin contribute to ET-1

mediated vasoconstriction not only in arterial disease [Wenzel *et al* 1998] but also in healthy blood vessels.

#### 11.3.1.1      **big ET-1<sub>[1-38]</sub>**

Given that big ET-1<sub>[1-38]</sub> has affinity for the endothelin A receptor which is 1000 fold less than ET-1<sub>[1-21]</sub> [Ishikawa *et al* 1994], and that big ET-1<sub>[1-38]</sub> is ~30 fold less potent than ET-1<sub>[1-21]</sub> it is unlikely that big ET-1<sub>[1-38]</sub> causes a major direct vasoconstrictor action and suggests that there is some conversion of big ET-1 to ET-1<sub>[1-21]</sub> in the skin. It has previously been demonstrated that big ET-1<sub>[1-38]</sub> is vasoactive in human arteries [Haynes *et al* 1994b] but not veins [Haynes *et al* 1995] and that this vasoconstriction can be blocked by ECE inhibition [Haynes *et al* 1994]. The presence of ECE activity in the skin is further supported in this present study by the vasodilatory effects of phosphoramidon. This is also consistent with preliminary clinical studies where systemic ECE inhibition caused systemic vasodilatation and a reduction in blood pressure [de Voogd *et al* 2001]. In our study there appeared to be a transient vasoconstriction to phosphoramidon although this was not consistent and did not reach statistical significance. This observation may warrant further investigation.

#### 11.3.1.2      **ET-1<sub>[1-31]</sub>**

The novel finding that ET-1<sub>[1-31]</sub> is a vasoconstrictor is of interest, and the first evidence of its vasoactive properties *in vivo* in man. If ET-1<sub>[1-31]</sub> is converted to ET-1<sub>[1-21]</sub> by a non-ECE pathway and if this contributes importantly to ET-1 generation

then specific receptor blockade may offer greater functional activity than ECE inhibition. Alternatively, ET-1<sub>[1-31]</sub> may have vasoconstricting activity of its own at endothelin receptors. Recently, the vasoconstricting effects of ET-1<sub>[1-31]</sub> have been shown to be mediated via the ET<sub>A</sub> receptor in rabbit renal resistance vessels [Ozawa 2003], an effect which was unaffected by phosphoramidon. Although ET-1<sub>[1-31]</sub> is less potent than ET-1<sub>[1-21]</sub>, as a vasoconstrictor, local production of ET-1<sub>[1-31]</sub> may occur specifically in tissues that express human chymase, such as from the mast cells within the shoulder region of coronary atheromatous plaques [Kaartinen *et al* 1994, Kovanen *et al* 1995]. Therefore, generation of ET-1<sub>[1-31]</sub> could contribute to coronary artery spasm at the time of plaque rupture. Indeed, ET-1<sub>[1-31]</sub> may be cleaved to ET-1<sub>[1-21]</sub> for its biological activity in cultured bronchial smooth muscle cells [Hayasaki-Kajiwara *et al* 1999] and in both guinea-pig [Honore *et al* 2002] and human arteries [Maguire *et al* 2001].

#### 11.3.1.3 ET antagonists

Endothelin antagonists have been studied in the skin by other groups. The selective ET<sub>A</sub> receptor antagonist PD147953 and the non-selective endothelin receptor antagonist PD145065 caused vasodilatation and attenuated the vasoconstrictor effects of ET-1<sub>[1-21]</sub> in the human skin *in vivo* [Wenzel *et al* 1994 & 2001]. BQ-123 is a selective ET<sub>A</sub> receptor antagonist and has been shown in many vascular beds [Berrazueta *et al* 1997] to be a vasodilator by blocking the effects of ET-1<sub>[1-21]</sub>. The data from Chapter 7 demonstrates that BQ-123 causes vasodilatation, confirming that ET-1 contributes to the maintenance of basal vascular tone in the skin of healthy volunteers. BQ-788 also caused skin vasodilatation. This result was unexpected as



selective ET<sub>B</sub> receptor antagonism causes vasoconstriction in skeletal muscle [Verhaar *et al* 1998] and when given systemically [Strachan *et al* 1999]. In addition, previous data in the skin microcirculation have demonstrated that ET-1 mediates vasoconstriction via the ET<sub>A</sub> receptor with little contribution from the ET<sub>B</sub> receptor [Wenzel *et al* 2001, Lipa *et al* 1999]. However, our data indicate that the dominant effect of ET-1<sub>[1-21]</sub> on the ET<sub>B</sub> receptor is vasoconstriction in the human skin microcirculation, and that blockade of the ET<sub>B</sub> receptor results in vasodilatation. It is possible that some of this effect may be a result of displacement of ET-1 from ET<sub>B</sub> receptors and a reduction of available binding sites, thereby increasing vasoconstriction via the ET<sub>A</sub> receptor.

Endothelin receptor antagonists have proven benefits in patients with primary pulmonary hypertension [Channick *et al* 2001] and reduce blood pressure in hypertensive patients [Krum *et al* 1998]. To date, clinical trials investigating the potential benefits of endothelin receptor antagonists in CHF have been disappointing [Coletta *et al* 2002]. ECE inhibitors inhibit endothelin production and may provide a potential alternative to endothelin receptor antagonism. Unlike receptor blockade which can increase plasma ET-1<sub>[1-21]</sub> concentrations, ECE inhibitors have the advantage that they block ET-1<sub>[1-21]</sub> production, thus reducing plasma ET-1<sub>[1-21]</sub> concentrations and leaving clearance receptors unblocked. However, the effects of raised plasma concentration of ET-1<sub>[1-21]</sub>, associated with ET<sub>B</sub> receptor blockade [Verhaar *et al* 1998], may not be relevant if ET<sub>A</sub> receptors are also blocked. However, if there are clinically significant non-ECE pathways of production with

vasoactive intermediary peptides such as ET-1<sub>[1-31]</sub> then ECE inhibition may be a less attractive treatment strategy.

In summary, the discovery of a vasoactive intermediary endothelin peptide may have importance for the further development of endothelin blockers as clinical therapies. Further studies are required to determine whether ET-1<sub>[1-31]</sub> is vasoactive in other vascular beds.

#### **11.4 Endothelin antagonists in cardiovascular disease**

While the dual ET<sub>A/B</sub> receptor antagonist bosentan has been investigated in patients with essential hypertension and shown to have sustained blood pressure lowering effects, it has not gained a licence for clinical use, probably due to concerns regarding liver toxicity and the range of alternative vasodilators of proven efficacy in this patient group. However, bosentan does have a licence for the treatment of primary pulmonary hypertension. Primary pulmonary hypertension has a poor prognosis and current treatment options are limited. However, morbidity benefits following treatment with Bosentan were recently demonstrated with a reduction in breathlessness and increased walking distances.

##### **11.4.1 Chronic heart failure: selective vs dual blockade**

Chronic heart failure is characterised by increased peripheral vasoconstriction and by increased concentrations of big ET-1 and ET-1. There have been several studies in patients with heart failure investigating the effects of selective ET<sub>A</sub> and dual ET<sub>A/B</sub> receptor antagonists although no direct head to head comparisons. In Chapter 10,

acute haemodynamic differences were found between selective  $ET_A$  and dual  $ET_{A/B}$  receptor antagonism. Disappointingly, recent clinical trials were unable to demonstrate significant clinical benefit with longer term treatment with either selective  $ET_A$  or dual  $ET_{A/B}$  receptor antagonist. Again, the disappointing results in patients with CHF on standard therapy makes it unlikely that endothelin antagonists will ever be used for the general treatment of CHF. However, there may be subsets of patients who will benefit from endothelin blockade, particularly patients who may be intolerant of other neuroendocrine blockade such as ACE inhibitors or beta blockers. In addition, ETAs may be of use in patients in whom the endothelin system is particularly elevated or in patients with concomitant pulmonary hypertension. In Chapter 10, the acute haemodynamic effects of selective  $ET_A$  versus dual  $ET_{A/B}$  receptor antagonism were studied in patients with stable chronic heart failure on standard medication. There were significant hemodynamic differences between the responses to selective  $ET_A$  and dual  $ET_{A/B}$  receptor blockade in patients with chronic heart failure. Both selective  $ET_A$  and dual  $ET_{A/B}$  receptor blockade increased cardiac output and reduced mean arterial pressure and systemic vascular resistance. However, selective  $ET_A$  receptor blockade caused a greater increase in cardiac output and reduction in systemic vascular resistance than dual  $ET_{A/B}$  receptor blockade. In contrast, selective  $ET_A$  and dual  $ET_{A/B}$  blockade caused similar reductions in both pulmonary artery pressure and pulmonary vascular resistance. There was a greater reduction in pulmonary artery pressure with dual blockade compared with selective  $ET_A$  blockade after low dose infusion although this difference was not apparent after high dose infusion.

Comparing magnitude of response between different endothelin antagonists in different patient populations is difficult but these data have demonstrated in this head-to-head study that selective endothelin blockade had greater effects on the systemic vasculature than dual endothelin antagonism.

#### **11.4.2 The ET<sub>B</sub> receptor is a clearance receptor**

There is increasing evidence that the ET<sub>B</sub> receptor has a role in the clearance of plasma ET-1. In keeping with previous work, selective ET<sub>A</sub> receptor blockade had no effect on plasma ET-1 concentrations whereas they were increased by dual blockade (Chapter 10). Because there was no change in plasma big ET-1 concentration, elevation of plasma ET-1 is likely to reflect interference with its clearance rather than an increase in its synthesis and release. Thus, selective ET<sub>A</sub> blockade has a theoretical benefit of leaving the endothelin clearance receptor (ET<sub>B</sub>) functional. Nevertheless, if the ET<sub>A</sub> receptor is also effectively blocked, during ET<sub>B</sub> receptor blockade, then the high circulating concentrations of ET-1 may not be of clinical importance.

#### **11.4.3 Clinical implications of selective ETA vs ETA/B blockade**

Although selective ET<sub>A</sub> receptor blockade had greater effects on systemic vascular resistance, there may be clinical situations in which blockade of the ET<sub>B</sub> receptor is desirable. There is a higher density of ET<sub>B</sub> receptors in the pulmonary vasculature, and these may be upregulated in pulmonary arterial hypertension [McCulloch *et al* 1995]. Also, endothelin-1 release across the pulmonary vascular bed correlates strongly with the pulmonary vascular resistance in chronic heart failure [Tsutamoto

*et al* 1994]. Raised pulmonary artery pressure is an independent risk factor in chronic heart failure and responds poorly to conventional therapies. Here, we have demonstrated that both selective ET<sub>A</sub> and dual ET<sub>A/B</sub> receptor blockade reduced pulmonary artery pressures.

Endothelin antagonism might, therefore, be of benefit in patients with heart failure and raised pulmonary artery pressures. Indeed, the dual antagonist, bosentan, has recently been approved for use in the treatment of primary pulmonary arterial hypertension based on its effectiveness in this situation. The long-term clinical effects of endothelin receptor blockade in patients with pulmonary hypertension secondary to chronic heart failure are unknown but it is tempting to speculate that it may also be more effective in this setting. We have failed to demonstrate convincingly whether there are true haemodynamic differences between selective ET<sub>A</sub> and dual ET<sub>A/B</sub> receptor antagonism in the pulmonary circulation. However, none of the patients in the present study had significant pulmonary hypertension. We believe that the role of endothelin antagonism now warrants further careful assessment in a much larger trial of patients with both heart failure and a significant degree of pulmonary hypertension.

The clinical impact of these haemodynamic changes is, of course, uncertain and can only be clarified in the context of large-scale clinical outcome studies. In Chapter 10 it was shown that selective ET<sub>A</sub> blockade caused more marked systemic vasodilatation than dual ET<sub>A/B</sub> receptor blockade in NYHA grade II/III patients with heart failure. To date, there have been only two, as yet unpublished, large scale,

randomized controlled trials of endothelin receptor blockade in patients with heart failure (NYHA grade III/IV), both of which have demonstrated no major clinical benefit of either bosentan ( $ET_A : ET_B$  selectivity  $\sim 10$ ) [Coletta *et al* 2002] or darusentan ( $ET_A : ET_B$  selectivity  $> 500$ ) [Luscher *et al* 2002].

In this study both selective  $ET_A$  and dual  $ET_{A/B}$  blockade cause acute systemic and pulmonary haemodynamic changes in patients with heart failure. However, important differences exist and selective  $ET_A$  blockade causes greater systemic haemodynamic effects than dual  $ET_{A/B}$  blockade. If ET antagonists are to have a future as therapies, there is now a need for further trials to determine whether selective or dual endothelin antagonism has greater potential efficacy in the treatment of patients with heart failure. Indeed there may be subsets of patients with differential activation of neurohormonal systems which would show greater benefit from endothelin blockade.

#### **11.4.4 $ET-1_{[1-31]}$ in patients with chronic heart failure**

Plasma big ET-1 concentrations are elevated in patients with CHF and correlate with disease severity [Wei *et al* 1994]. In Chapter 7 plasma from a different cohort of patients with heart failure was compared with age and sex matched controls. Plasma concentrations of big ET-1 were found to be elevated, consistent with these prior data [Wei *et al* 1994], although plasma  $ET-1_{[1-21]}$  concentrations were not elevated. This was unexpected as plasma  $ET-1_{[1-21]}$  concentrations are also reported to be elevated in patients with chronic heart failure [Wei *et al* 1994]. However, big  $ET-1_{[1-38]}$  plasma concentrations are a more reliable marker of chronic heart failure and, in our study, these were increased by  $\sim 2$  fold. We have also recruited stable patients

who were well controlled on medical therapy. It is likely, therefore, that our patient population had less neurohormonal activation and may have had less generation of big ET-1<sub>[1-38]</sub> than those enrolled into previous studies.

In patients with chronic heart failure, there does not appear to be a general increase in generation of ET-1<sub>[1-31]</sub>. This study does not however exclude the possibility of local increases in ET-1<sub>[1-31]</sub> production, such as within areas of inflammation. Moreover, local ET-1<sub>[1-31]</sub> generation may be specific to those tissues with increased chymase activity such as failing human myocardium [Urata *et al* 1990]. In addition, increased chymase activity is seen in mast cells at the shoulder regions of coronary atheromatous plaques [Kovanen *et al* 1995, Kaartinen *et al* 1994], and ET-1<sub>[1-31]</sub> may be implicated in the aetiology of coronary artery spasm at the time of plaque rupture. Further studies are clearly required to determine how ET-1<sub>[1-31]</sub> is processed and to identify situations where increased local ET-1<sub>[1-31]</sub> generation may occur.

In conclusion, data in Chapter 7 have shown that plasma ET-1<sub>[1-31]</sub> concentrations can be measured reliably but, in contrast to big ET-1<sub>[1-38]</sub>, do not appear to be elevated in patients with heart failure. These data suggest that ET-1<sub>[1-31]</sub> may not be useful as a predictive test and that generation is not sufficient to increase circulating plasma concentrations in patients with stable chronic heart failure.

However, current assessment of patients with heart failure does not include detailed neuroendocrine assessment and whether there are subsets of patients with preferential activation of either endothelin, angiotensin or beta-adrenergic systems is

unknown. Indeed, these systems interact in a complex manner which may not be the same in different patients groups. In Chapter 8, data from isolated human myocardium demonstrated that there may be antagonism between the endothelin and beta-adrenergic stimulation. Thus, in certain situations, elevated endothelin concentrations may actually be protective, and associations between severity and prognosis in heart failure may represent associations due to beneficial adaptations rather than a causal link.

### **11.5 Effects of the ET and beta-adrenergic system in the heart**

In Chapter 8, interaction between the endothelin and beta-adrenergic system were investigated and the results discussed. This study confirmed that ET-1 and beta-adrenergic stimulation increases force of contraction in isolated human myocardium, but that ET antagonism does not appear to affect basal tone in isolated myocardium, suggesting either that there is no resting ET-1 mediated inotropy or that this mechanism is saturated at baseline. We have observed, for the first time, that ET-1 functionally attenuates the inotropic effects of isoprenaline in human myocardium. This reduction in force was observed after maximal stimulation of the beta-adrenergic system. The underlying mechanism is likely to be interaction at the level of adenylate cyclase as seen in isolated membrane studies where ET-1 was shown to inhibit the effect of beta<sub>1</sub>-adrenoceptor activation on this enzyme.

These results suggest that, in the human myocardium, ET-1 functionally antagonises the effects of beta-adrenergic stimulation. However, care should be taken when interpreting these results as supra-physiological concentrations of agonists were



used, although, it appears that there is functional antagonism between these two hormone systems, presumably at the level of receptor, or post receptor cross-talk.

### **11.5.1 Clinical implications**

The results of this study may have important consequences in conditions such as CHF where both ET-1 and the beta-adrenergic systems are up-regulated and drugs blocking their actions are being developed. Catecholamine release and therefore beta-adrenergic stimulation is increased in CHF and also in episodes of acute heart failure and acute coronary syndromes. This can lead to increases in arrhythmias and sudden cardiac death, the most common cause of death in patients with CHF. In CHF there is a reduced responsiveness to beta-agonists, probably due to combination of reduced receptor number and post receptor changes. It could be that some of these changes are due to an interaction with the ET system which is also up-regulated in CHF. The importance of this interaction may be that ET-1 while a marker of disease severity in CHF actually protects against catecholamine stimulation. It is known that catecholamine blockade with drugs such as beta-blockers confers mortality and morbidity benefits. With the possible future introduction of ETARs for the treatment of CHF, the physiological and patho-physiological interactions between these two systems is of importance. Indeed, the first trial of endothelin antagonist in patients with heart failure, many of whom were on beta blocker treatment was negative, demonstrating no mortality benefit [Coletta *et al* 2002, Luscher *et al* 2002]. Clearly these experiments require further investigation, but beyond the scope of simple isometric force contraction experiments.

## 11.6 Endothelin in 'predisease' states

Results from the trials of endothelin antagonists in patients with clinical disease such as chronic heart failure have been disappointing. However, data in Chapter 9 suggest that there may be a role for modulation of the endothelin system in high risk patients or 'pre-disease' states such as hypercholesterolaemia.

This study demonstrated that 8 weeks of statin therapy caused a trend towards a reduction in arterial stiffness and increased vasodilatory responses to selective ET<sub>A</sub> receptor blockade. There were no differences in responses to selective ET<sub>B</sub> receptor or combined ET<sub>A/B</sub> receptor between the placebo and statin treatment periods.

Consistent with studies in healthy volunteers, selective ET<sub>A</sub> receptor blockade with BQ-123 increased forearm blood flow. However, there was a trend towards an increase in this vasodilation after treatment with cerivastatin. These preliminary results are of interest. If the balance of effect at the ET<sub>B</sub> receptor is indeed vasodilatation then the vasodilatation seen with ET<sub>A</sub> receptor blockade may have two components, firstly the reduction in ET-1 tone mediated through the ET<sub>A</sub> receptor when blocked and secondly there will be an increase in ET<sub>B</sub> receptor mediated vasodilatation due to increased availability of ET-1 due to ET-1 displacement and reduction in the number of target receptors. The trend towards an increase in ET<sub>A</sub> mediated vasodilatation following cerivastatin would support the hypothesis that statin therapy increases NO mediated vasodilatation via the ET<sub>B</sub> receptor, given that there is no difference between groups when the ET<sub>B</sub> receptor is concomitantly

blocked by combine BQ123 and BQ788 infusion. It has been previously shown that hypercholesterolaemia causes an impairment of NO mediated vasodilation and that this can be at least partially reversed by lipid lowering therapy [O'Driscoll *et al* 1997]. However, there may also be structural changes to the arterial wall which would be less likely to be reversed after 8 weeks of lipid lowering and therefore a longer treatment period may be expected to have greater effects. The potential interaction between endothelin antagonists and statin therapy is of interest and deserves further investigation.

In summary, investigation of the endothelin system has been disappointing in terms of identifying groups of patients who would benefit from endothelin antagonism. Except for primary pulmonary hypertension, endothelin antagonists have failed to find a clinical niche. However, there is much focus on prevention of clinical disease and endothelin antagonists may yet find an important role in clinical medicine.

### **11.7 Future directions**

Endothelin antagonists have not fulfilled their expected potential. Other than in primary pulmonary hypertension they have not yet found a role in therapeutics. While endothelin antagonists have been shown to reduce blood pressure in man and modify atherosclerosis in animal models, initial clinical studies in patients with cardiac disease (eg chronic heart failure) have been disappointing. Nevertheless, the endothelin system is activated in a variety of conditions such as hypertension, diabetes and renal failure and its blockade may yet prove of benefit in a subgroup of patients

The work presented in this thesis demonstrates that there are functionally active endothelin intermediaries (ET-1<sub>[1-31]</sub>), as well as potentially important interactions between the endothelin system and other neurohormal systems (beta-adrenergic). Furthermore, differences exist between the effects of selective and dual endothelin receptor blockade in patients (chronic heart failure). These findings may guide future research involving the endothelin system. However, whether manipulation of the endothelin system will prove to be of benefit requires further large-scale trials in specific patient groups.

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## CARDIOVASCULAR MEDICINE

## Direct comparison of selective endothelin A and non-selective endothelin A/B receptor blockade in chronic heart failure

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**Objective:** To investigate the potential differential effects of selective endothelin (ET) A and dual ET-A/B receptor blockade in patients with chronic heart failure.

**Methods:** Nine patients with chronic heart failure (New York Heart Association class II-III) each received intravenous infusions of BQ-123 alone (selective ET-A blockade) and combined BQ-123 and BQ-788 (dual ET-A/B blockade) in a randomised, placebo controlled, three way crossover study.

**Results:** Selective ET-A blockade increased cardiac output (maximum mean (SEM) 33 (12)%,  $p < 0.001$ ) and reduced mean arterial pressure (maximum -13 (4)%,  $p < 0.001$ ) and systemic vascular resistance (maximum -26 (8)%,  $p < 0.001$ ), without changing heart rate ( $p = 0.38$ ). Dual ET-A/B blockade significantly reduced the changes in all these haemodynamic variables compared with selective ET-A blockade ( $p < 0.05$ ). Selective ET-A blockade reduced pulmonary artery pressure (maximum 25 (7)%,  $p = 0.01$ ) and pulmonary vascular resistance (maximum 72 (39)%,  $p < 0.001$ ). However, there was no difference between these effects and those seen with dual ET-A/B blockade. Unlike selective ET-A blockade, dual ET-A/B blockade increased plasma ET-1 concentrations (by 47 (4)% with low dose and 61 (8)% with high dose, both  $p < 0.05$ ).

**Conclusions:** While there appeared to be similar reductions in pulmonary pressures with selective ET-A and dual ET-A/B blockade, selective ET-A blockade caused greater systemic vasodilatation and did not affect ET-1 clearance. In conclusion, there are significant haemodynamic differences between selective ET-A and dual ET-A/B blockade, which may determine responses in individual patients.

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Endothelin (ET) 1 is a potent endogenous vasoconstrictor in humans and contributes to the maintenance of basal vascular tone<sup>1</sup> and blood pressure<sup>2</sup> in healthy people and patients with systemic arterial hypertension.<sup>3,4</sup> It acts through two receptor subtypes: the ET-A and ET-B receptors. While both receptors are expressed on vascular smooth muscle cells and mediate vasoconstriction, only the ET-B receptor is located on the endothelium, where it produces a prostanoid and nitric oxide mediated vasodilatation. Thus, ET-B receptor mediated effects are complex and include vasoconstriction, endothelium dependent vasodilatation, and a role in the clearance of ET-1.<sup>5</sup> In healthy people, in contrast to the vasodilator and vasodepressor effects of ET-A receptor blockade,<sup>6,7</sup> systemic ET-B receptor blockade has vasoconstrictor and pressor effects,<sup>8</sup> suggesting that the vascular balance of basal ET-B receptor activation favours vasodilatation.

Chronic heart failure is associated with neurohumoral activation as a consequence of reductions in cardiac reserve, systemic blood pressure, and renal perfusion. This leads to peripheral vasoconstriction, increased systemic vascular resistance, and sodium and water retention, which together increase cardiac work and further compromise cardiac performance. Many regulatory mechanisms are involved in this maladaptive response, including the renin-angiotensin, sympathetic nervous, and vasopressin systems. The ET system also appears to contribute to the pathophysiology of heart failure, which is associated with increased plasma ET-1 concentrations<sup>9,10</sup> that correlate with haemodynamic changes,<sup>11,12</sup> reduced exercise capacity,<sup>13</sup> and a poor prognosis.<sup>14</sup>

In patients with chronic heart failure, systemic administration of both selective ET-A<sup>15-17</sup> and non-selective ET-A/B

blockade<sup>18-21</sup> reduces systemic vascular resistance and increases cardiac output. In patients with acute decompensated heart failure systemic administration of non-selective ET-A/B blockade has been shown to have beneficial effects.<sup>22</sup> However, systemic ET-B blockade increases systemic vascular resistance and has potentially detrimental effects in patients with chronic heart failure.<sup>23</sup> Therefore, the question arises as to whether differences between selective ET-A and non-selective ET-A/B blockade can influence haemodynamic responses to ET blockade, which in turn might have contributed to the recent failure of ET blockade as a treatment approach for patients with chronic heart failure.<sup>24,25</sup> However, to date, there have been no direct studies comparing these two approaches.

The objectives of this placebo controlled study, in patients with stable chronic heart failure, were to compare in a head to head manner the effects of selective ET-A blockade with non-selective ET-A/B blockade on systemic and pulmonary haemodynamic function.

## METHODS

## Patient selection

Nine patients with chronic heart failure (New York Heart Association (NYHA) class II-III) caused by left ventricular dysfunction were recruited if they had an ejection fraction  $\leq 35\%$  (by echocardiography with the biplanar Simpson's rule) and had been stable with treatment, including angiotensin converting enzyme inhibitor or angiotensin receptor antagonist, for at least three months. Patients were all in sinus rhythm and no patient had a pacemaker or implantable cardiac defibrillator. Patients were excluded if they had insulin dependent diabetes mellitus, abnormal liver function, renal impairment (creatinine  $> 200 \mu\text{mol/l}$  for

men; > 180 µmol/l for women) or a systolic blood pressure > 190 or < 90 mm Hg, or within three months had undergone coronary artery bypass graft surgery or percutaneous coronary intervention or had an acute coronary syndrome, myocardial infarction, or cerebrovascular accident.

The study was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient before entry into the study.

Measurements

Blood pressure and heart rate were measured non-invasively with a Dynamap compact TS (Critikon LLC, Ascot, UK). Cardiac output, mean pulmonary artery pressure, pulmonary artery wedge pressure, and central venous pressure were measured continuously with a single multilumen thermolulution cardiac output pulmonary artery catheter (Swan-Ganz CCombo—CCO/SVO2; Edwards Lifesciences, Irvine, California, USA). Cardiac output was calculated automatically (Vigilence, Edwards Critical Care, Baxter's Healthcare Corporation, Irvine, California, USA) and, at each time point, the cardiac output was taken as the mean of three measurements.

Protocol

All patients attended fasted at 7.30 am on three occasions at least one week apart. Patients were asked to omit their regular medications on the morning of the study. The studies were conducted in a quiet, draught-free room maintained at a constant temperature (22–24°C). A pulmonary artery catheter was inserted through a 9 French femoral venous sheath into the right pulmonary artery and was flushed with 0.9% heparinised saline. Before starting drug administration, patients underwent an equilibration period of > 90 minutes until blood pressure, heart rate, and cardiac output were stable, with three consecutive measurements within 10%. Study drugs were administered by 15 minute infusion in two incremental doses 60 minutes apart.

Drug administration

A venous cannula for drug administration was inserted under local anaesthesia. Pharmaceutical grade BQ-123 and BQ-788 (Cinalfa AG, L  ufelfingen, Switzerland) were dissolved in 0.9% saline (Baxter Healthcare Ltd, Thetford, UK). On each study day, patients received a low dose infusion at t = 0 for 15 minutes followed by a high dose infusion at t = 60 minutes for 15 minutes. On different study days and in

random order, patients received saline placebo, BQ-123 (low dose, 1.5 µmol; high dose, 15 µmol) alone or the co-infusion of BQ-123 (low dose, 1.5 µmol; high dose, 15 µmol) and BQ-788 (low dose, 0.45 µmol; high dose, 4.5 µmol). These doses were selected following studies in healthy volunteers given BQ-123 and BQ-788. This dose of BQ-123 was sufficient to reduce systemic vascular resistance and block the effects of local infusion of ET-1 into the forearm.<sup>7</sup> This dose of BQ-788 was sufficient to reduce systemic vascular resistance.<sup>8</sup> The detailed rationale for these doses has been discussed elsewhere.<sup>26</sup>

Blood sampling and plasma assays

Venous blood for ET-1 and big ET-1 (the 38 amino acid precursor of ET-1) assay was taken from the femoral vein. Blood was collected into 0.16% EDTA (Sarstedt, Aktiengesell Schaft & Co, Germany) and immediately separated by centrifugation (2500 g for 20 minutes at 4°C) and stored at –80°C until analysis. Following extraction in Bond Elut columns (Varian, Harbor City, California, USA), ET-1 (Peninsula Laboratories Europe Ltd, St Helens, UK) and big ET-1 (Peninsula Laboratories Europe Ltd) concentrations were determined by radioimmunoassay as previously described.<sup>27</sup> The intra-assay coefficients of variability were 7.0 and 7.2%, respectively, and the interassay coefficients of variability were 9.0 and 9.3%, respectively.

1

Table 2 Baseline parameters

Parameter	Placebo (n=9)	BQ-123 (n=9)	BQ-123 + BQ-788 (n=9)
HR (beats/min)	64 (5)	62 (4)	63 (5)
MAP (mm Hg)	91 (7)	87 (6)	90 (7)
SBP (mm Hg)	106 (8)	104 (9)	106 (9)
DBP (mm Hg)	69 (4)	68 (6)	71 (4)
CVP (mm Hg)	5 (1)	5 (1)	3 (2)
CO (l/min)	5.0 (0.3)	5.5 (0.4)	5.3 (0.3)
SVR (dyn.s/cm <sup>-5</sup> )	1462 (150)	1266 (138)	1344 (138)
MPAP (mm Hg)	16 (1)	16 (1)	17 (2)
PAWP (mm Hg)	11 (2)	11 (1)	12 (3)
PVR (dyn.s/cm <sup>-5</sup> )	83 (12)	91 (14)	78 (18)

Data are mean (SEM).  
CO, cardiac output; CVP, central venous pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PVR, pulmonary vascular resistance; SBP, systolic blood pressure; SVR, systemic vascular resistance.

Table 1 Patient characteristics and medications

Patient	Age (years)	BMI (kg/m <sup>2</sup> )	BP (mm Hg)	HR (beats/min)	MPAP (mm Hg)	PAWP (mm Hg)	Cause of HF	NYHA	Drugs and dose*
1	75	26	186/79	52	20	11	Ischaemic	III	ASA75, En20, Bu1, Sim20
2	63	23	120/74	64	13	6	Idiopathic	II	ASA75, En20
3	60	27	122/58	62	17	12	Ischaemic	III	Val80, Frus40
4	43	30	98/64	77	12	8	Ischaemic	II	ASA75, Lis10, Frus40
5	56	29	139/88	96	11	6	Ischaemic	III	ASA75, Lis20, Frus40, Car12.5, Dig250, Sim20
6	61	26	136/77	66	16	12	Ischaemic	III	ASA75, Lis10, Frus40, Spir25, Dig125, Sim20
7	52	36	184/106	94	25	23	Ischaemic	III	Lis15, Frus20, Aten100
8	74	33	143/75	58	14	10	Ischaemic	III	ASA75, Lis10, Frus40, Bis10, Sim10
9	67	21	154/78	65	14	10	Ischaemic	II	ASA75, Lis10, Aten50, Pra20
Average	61	28	142/78	70	16	11			
SEM	3	1	9/4	5	1	2			

\*Doses in mg except for digoxin (µg).  
ASA, aspirin; Aten, atenolol; Bis, bisoprolol; BMI, body mass index; BP, blood pressure; Bu, bumetanide; Car, carvedilol; Dig, digoxin; En, enalapril; Frus, furosemide/amiloride; Frus, furosemide; HF, heart failure; HR, heart rate; Lis, lisinopril; MPAP, mean pulmonary artery pressure; NYHA, New York Heart Association; PAWP, pulmonary artery wedge pressure; Pra, pravastatin; Sim, simvastatin; Spir, spironolactone; Val, valsartan.



## Data and statistical analyses

Data are expressed as mean (SEM) change from baseline or mean (SEM) area under the curve (AUC) unless otherwise specified. Data were examined by analysis of variance with repeated measures over time and Student's *t* test with correction for multiple measures where appropriate (Excel version 5.0, Microsoft, Redmond, Washington, USA). Significance was taken at the 5% level.

## RESULTS

Table 1 shows baseline patient characteristics and medications. There were no adverse events and the study was well tolerated by all patients. There were no significant differences in baseline haemodynamic variables between study visits (table 2). Placebo administration caused no significant changes in haemodynamic variables throughout the course of the study (analysis of variance  $p > 0.9$ ).

### Cardiac output and heart rate

In comparison with placebo, BQ-123 alone (AUC  $p < 0.001$ ), but not BQ-123/788 (AUC  $p = 0.08$ ), increased cardiac output with a maximum increase of 33 (12)% at 75 minutes. Infusion of BQ-123 alone increased cardiac output compared with BQ-123/788 (AUC  $p < 0.001$ ) (fig 1C, fig 2). There was no significant change in heart rate with either BQ-123 alone (AUC  $p = 0.38$ ) or BQ-123/788 (AUC  $p = 0.39$ ) (fig 1A, fig 2).

### Left ventricular filling pressure and systemic haemodynamic variables

In comparison with placebo, BQ-123 alone (AUC  $p = 0.01$ ) and BQ-123/788 (AUC  $p < 0.01$ ) reduced pulmonary artery wedge pressure by a maximum of 19 (7)% at 150 minutes and 26 (7)% at 105 minutes, respectively (fig 2, fig 3C). There was no difference between the magnitude of reduction in

pulmonary artery wedge pressure between BQ-123 alone and BQ-123/788 (AUC  $p = 0.47$ ). BQ-123 alone (AUC  $p < 0.001$ ) and BQ-123/788 (AUC  $p < 0.05$ ) reduced mean arterial pressure by a maximum of 14 (5)% and 12 (4)%, respectively, at 150 minutes. BQ-123 alone reduced mean arterial pressure to a greater degree than BQ-123/788 (AUC  $p < 0.05$ ) (fig 1B, fig 2).

BQ-123 alone (AUC  $p < 0.001$ ) and BQ-123/788 (AUC  $p < 0.05$ ) reduced systemic vascular resistance by a maximum of 26 (8)% and 16 (5)%, respectively, at 75 minutes in comparison with placebo. BQ-123 alone reduced systemic vascular resistance to a greater degree than BQ-123/788 (AUC  $p < 0.05$ ) (fig 1D, figs 2 and 3).

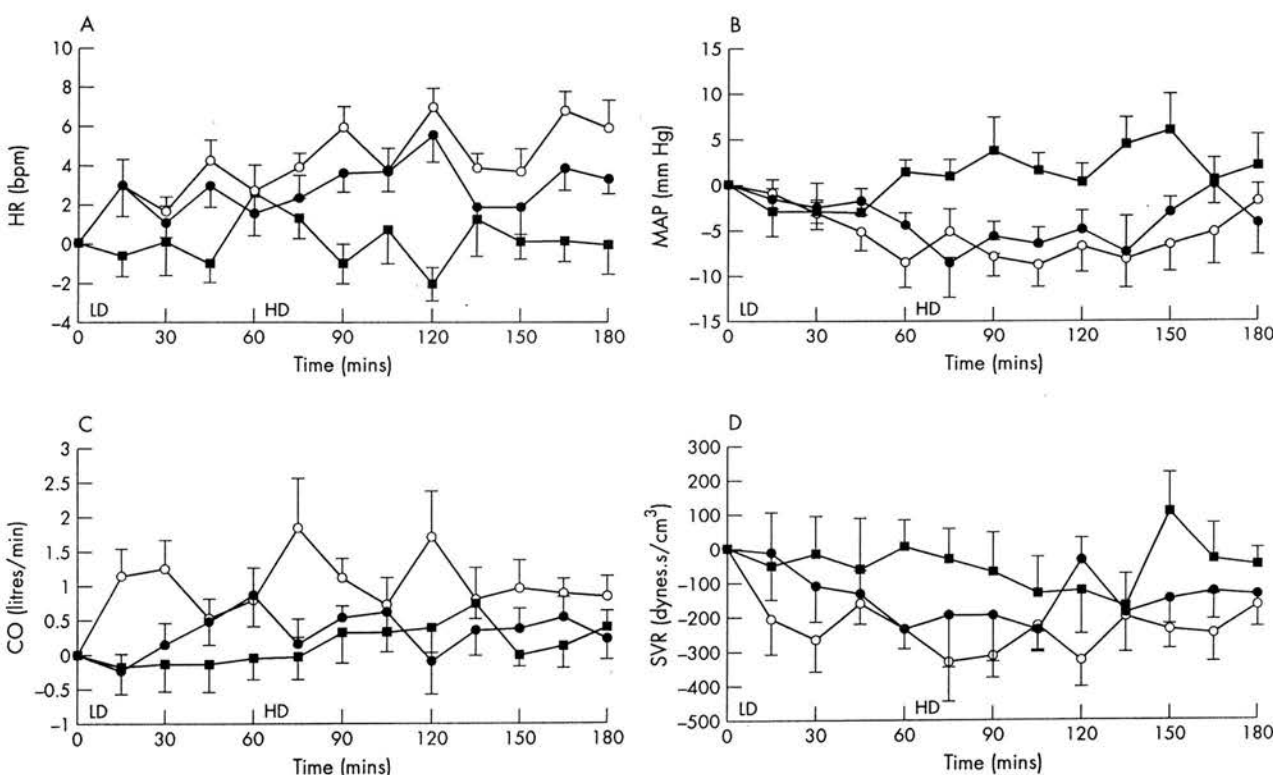
### Right ventricular filling pressure and pulmonary haemodynamic variables

In comparison with placebo, neither BQ-123 alone (AUC  $p = 0.17$ ) nor BQ-123/788 (AUC  $p = 0.69$ ) changed central venous pressure (fig 2, fig 3A). BQ-123 alone (AUC  $p = 0.01$ ) and BQ-123/788 (AUC  $p = 0.02$ ) reduced mean pulmonary arterial pressure by a maximum of 25 (7)% and 26 (6)%, respectively, at 90 minutes. There was no significant difference between these responses (AUC  $p = 0.98$ ) (fig 2, fig 3B).

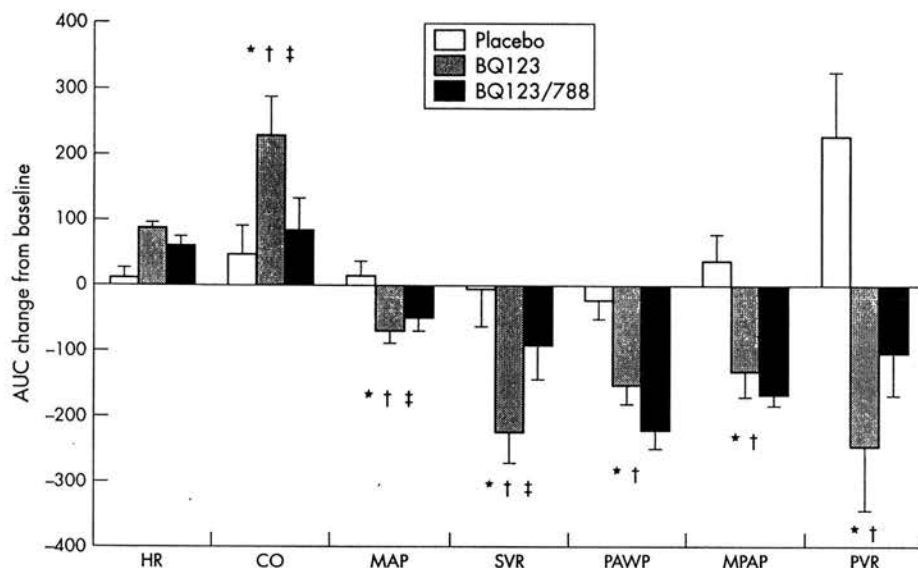
In comparison with placebo, both BQ-123 alone and BQ-123/788 (AUC both  $p < 0.001$ ) reduced pulmonary vascular resistance by a maximum of 72 (39)% and 40 (16)%, respectively, at 75 minutes. There was no significant difference between these responses (AUC  $p = 0.49$ ) (fig 2, fig 3D).

### Plasma ET-1 and big ET-1

There was no change in plasma concentrations of big ET-1 with placebo, BQ-123 alone, or BQ-123/788. There was no significant change in plasma ET-1 concentrations with



**Figure 1** Effect of selective endothelin (ET) A blockade (open circles), dual ET-A/B blockade (solid circles), and placebo (solid squares) on (A) heart rate (HR), (B) mean arterial pressure (MAP), (C) cardiac output (CO), and (D) systemic vascular resistance (SVR) at low dose (LD) and high dose (HD).



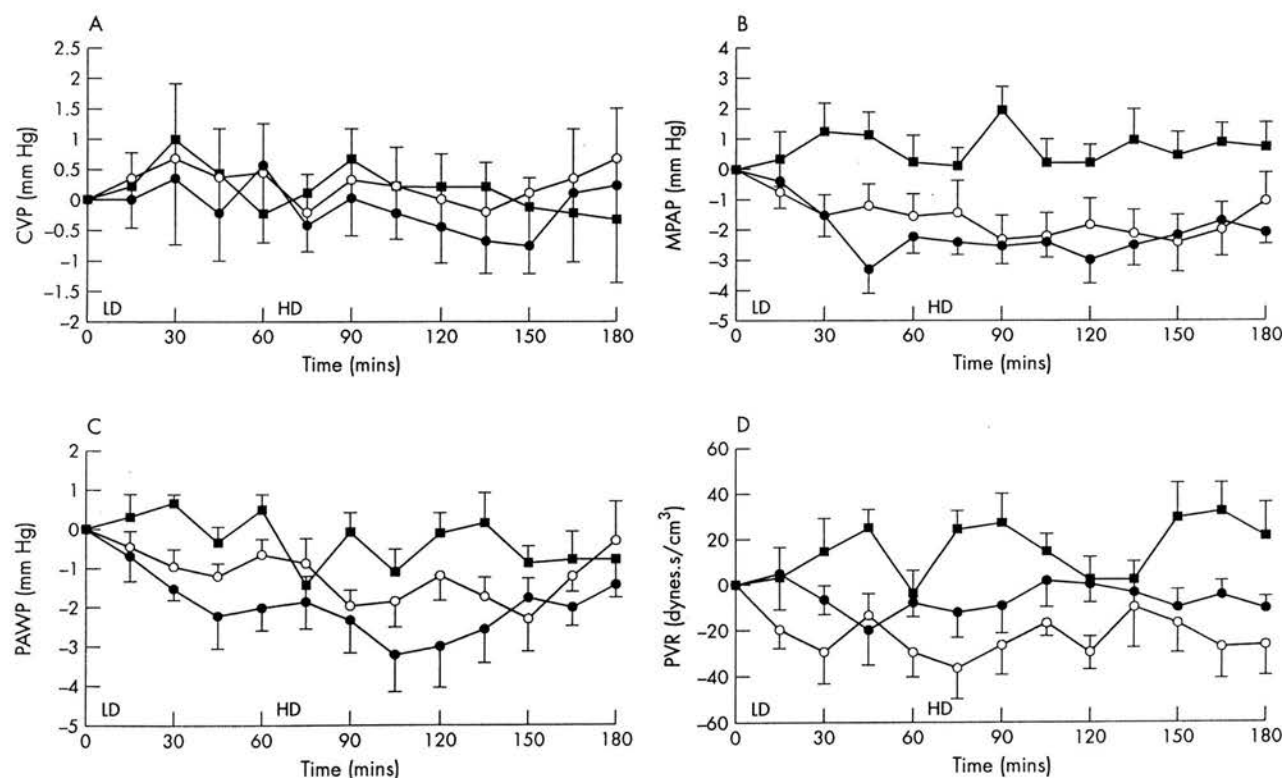
**Figure 2** Comparison of the haemodynamic effects of placebo (white), selective ET-A blockade (grey), and dual ET-A/B blockade (black) on HR, CO, MAP, SVR, pulmonary arterial wedge pressure (PAWP), mean pulmonary artery pressure (MPAP) and pulmonary vascular resistance (PVR). AUC, area under the curve. \* $p < 0.05$  BQ-123 v placebo; † $p < 0.05$  BQ-123/788 v placebo; ‡ $p < 0.05$  BQ-123 v BQ-123/788.

placebo or BQ-123 alone, whereas BQ-123/788 caused an increase in plasma ET-1 concentrations (47% with low dose and 61% with high dose, both  $p < 0.05$ ) (fig 4).

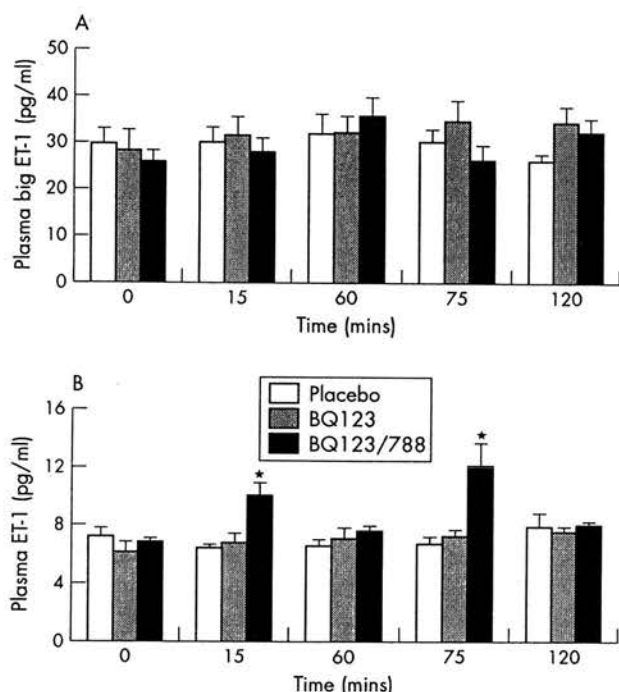
## DISCUSSION

In this randomised placebo controlled crossover study, we have shown, for the first time, that there are small but significant haemodynamic differences between the responses to selective ET-A and non-selective ET-A/B receptor blockade in patients with chronic heart failure. Both selective ET-A and non-selective ET-A/B receptor blockade increased cardiac

output and reduced mean arterial pressure and systemic vascular resistance. However, selective ET-A receptor blockade caused a greater increase in cardiac output and reduction in systemic vascular resistance than non-selective ET-A/B receptor blockade. In contrast, selective ET-A and non-selective ET-A/B blockade caused similar reductions in both pulmonary artery pressure and pulmonary vascular resistance. There was a greater reduction in pulmonary artery pressure with non-selective blockade than with selective ET-A blockade after low dose infusion, although this difference was not apparent after high dose infusion.



**Figure 3** Effect of selective ET-A blockade (open circles), dual ET-A/B blockade (solid circles), and placebo (solid squares) on (A) central venous pressure (CVP), (B) MPAP, (C) PAWP, and (D) PVR at low dose (LD) and high dose (HD).



**Figure 4** Effects of placebo (white), selective ET-A blockade (grey), and combined ET-A/B blockade (black) on plasma concentration of (A) big ET-1 and (B) ET-1. \*p < 0.05 v baseline.

This is the first study to directly compare systemic selective ET-A and non-selective ET-A/B blockade in patients with heart failure. Our findings are consistent with other short term studies, which have shown that both selective ET-A<sup>17</sup> and non-selective ET-A/B<sup>20-22</sup> blockade increase cardiac output and reduce systemic vascular resistance, importantly with no change in heart rate, and that both selective ET-A<sup>16, 17</sup> and non-selective ET-A/B<sup>20-22</sup> blockade reduce pulmonary vascular resistance and pulmonary artery wedge pressure. Comparing magnitude of response between different ET antagonists in different patient populations is difficult but we have shown in this head to head study that selective ET blockade had greater effects than non-selective ET antagonism on the systemic vasculature.

There is increasing evidence that the ET-B receptor has a role in the clearance of plasma ET-1. Plasma ET-1 concentration increases after systemic selective ET-B blockade in healthy people<sup>8</sup> and after non-selective ET-A/B blockade in healthy people<sup>28</sup> and in patients with hypertension<sup>4</sup> and with chronic heart failure.<sup>18-20</sup> However, the effects of systemic ET-A blockade alone are less consistent with little, if any, increase in plasma ET-1 concentrations in most studies,<sup>6, 15, 16</sup> although one study did report an increase at higher degrees of ET-A blockade.<sup>17</sup> These results are confirmed in our study, in which selective ET-A receptor blockade had no effect, whereas plasma ET-1 concentrations were increased by non-selective blockade. Because there was no change in plasma big ET-1 concentration, increased plasma ET-1 is likely to reflect interference with its clearance rather than an increase in its synthesis and release. Thus, selective ET-A blockade has a theoretical benefit of leaving the ET clearance receptor (ET-B) functional. Nevertheless, if the ET-A receptor is also effectively blocked during ET-B receptor blockade then the high circulating concentrations of ET-1 may not be of clinical importance.

Although selective ET-A receptor blockade had greater effects on systemic vascular resistance, there may be clinical situations in which blockade of the ET-B receptor is desirable.

There is a higher density of ET-B receptors in the pulmonary vasculature and these may be upregulated in pulmonary arterial hypertension,<sup>29</sup> though selective ET-A and non-selective ET-A/B receptor blockade have not yet been compared head to head in this condition. Also, ET-1 release across the pulmonary vascular bed correlates strongly with the pulmonary vascular resistance in chronic heart failure.<sup>10</sup> Raised pulmonary artery pressure is an independent risk factor in chronic heart failure and responds poorly to conventional treatments. Here, we showed that both selective ET-A and non-selective ET-A/B receptor blockade reduce pulmonary artery pressures.

These observations suggest that ET antagonism may benefit patients with heart failure who also have raised pulmonary artery pressures, although we did not directly address this condition in our study. Indeed, the non-selective antagonist bosentan has recently been approved to treat primary pulmonary arterial hypertension based on its effectiveness in this situation.<sup>31</sup> The long term clinical effects of ET receptor blockade in patients with pulmonary hypertension secondary to chronic heart failure are unknown, but it is tempting to speculate that ET receptor blockade may also be more effective in this setting. We have failed to show convincingly whether there are true haemodynamic differences between selective ET-A and non-selective ET-A/B receptor antagonism in the pulmonary circulation. However, none of the patients in the present study had significant pulmonary hypertension. We believe that the role of ET antagonism now warrants further careful assessment in a much larger trial of patients with both heart failure and a significant degree of pulmonary hypertension.

Many studies use agents that, while termed "selective" or "dual" inhibitors of ET-A and ET-B receptors, have a range of receptor selectivities, mostly inhibiting the ET-A receptor at much lower concentrations than at the ET-B receptor.<sup>26</sup> In this study we have used two receptor antagonists, BQ-123 and BQ-788, given separately and with selectivity for the ET-A and ET-B receptor, respectively. Therefore, it is important to recognise that we have examined mechanistically the influence of major blockade of the ET-B receptor on responses to full ET-A blockade. This may not exactly represent the clinical situation that exists with non-selective antagonists, such as bosentan, which are relatively selective for the ET-A receptor (ET-A:ET-B selectivity > 10). The doses of BQ-123 given here have been shown to produce maximum systemic haemodynamic effects and to block responses to forearm artery infusion of ET-1, but not to increase plasma ET-1 concentrations. Given that BQ-123 caused greater systemic vasodilatation than the combination with BQ-788, the overall haemodynamic effect of ET-B blockade in patients with heart failure is likely to be vasoconstriction, a finding consistent with other work.<sup>23, 32</sup>

As a limitation, this was an acute haemodynamic study and we have not assessed whether these effects are sustained in the long term. Nevertheless, previous haemodynamic studies indicate that the acute effects of both selective ET-A<sup>24</sup> and non-selective<sup>32</sup> ET receptor blockade are maintained, or even enhanced, over several weeks and therefore likely to be sustained. The clinical impact of these haemodynamic changes is, of course, uncertain and can only be clarified in the context of large scale clinical outcome studies. We have shown that selective ET-A blockade causes more major systemic vasodilatation than non-selective ET-A/B receptor blockade in New York Heart Association (NYHA) class II-III patients with heart failure. To date, there have been only two as yet unpublished large scale, randomised controlled trials of ET receptor blockade in patients with heart failure (NYHA class III-IV), both of which observed no major clinical benefit of either bosentan (ET-A:ET-B selectivity ~10)<sup>25</sup> or darusen-



tan (ET-A:ET-B selectivity > 500).<sup>24</sup> The results of these longer term studies were disappointing, although perhaps it is not surprising that two agents with 10 to > 500 selectivity for the ET-A receptor yielded similar results given the small haemodynamic differences found in the current study, when much greater relative ET-B receptor blockade was achieved. Nevertheless, bosentan has found utility in the treatment of primary pulmonary hypertension and whether it may have utility in a subset of patients with CHF with secondary pulmonary hypertension remains to be seen.

### Conclusions

In this study both selective ET-A and non-selective ET-A/B blockade cause acute systemic and pulmonary haemodynamic changes in patients with heart failure. However, differences exist and selective ET-A blockade causes greater systemic haemodynamic effects than non-selective ET-A/B blockade.

### ACKNOWLEDGEMENTS

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# The Effect of Cerivastatin Therapy on Vascular Responses to Endothelin Antagonists in Humans

Stephen J. Leslie, James C. Spratt, Lynn Grieg, Teresa Attina, Martin A. Denvir, and David J. Webb

**Abstract:** Endothelin blocking drugs have vasodilator effects mediated at least in part via the nitric oxide system. Hypercholesterolaemia is associated with vascular dysfunction manifest as impaired nitric oxide-mediated vasodilatation and arterial stiffness. Treatment with HMG CoA reductase inhibitors (statins) has proven mortality benefits in a range of patient populations. Subjects ( $n = 5$ ) received either placebo or 800  $\mu\text{g}$  cerivastatin for an 8-week period in a double-blind, placebo-controlled, cross-over study. Cerivastatin reduced the total plasma cholesterol compared with baseline by 27% ( $5.4 \pm 0.4$  mmol/L versus  $7.3 \pm 0.4$  mmol/L,  $P = 0.04$ ). Selective endothelin-A receptor blockade caused an increase in forearm blood flow (FBF) ( $18.0 \pm 7.2\%$ ,  $P = 0.04$ ). Compared with placebo, cerivastatin therapy caused a trend towards a further increase in FBF ( $18.0 \pm 7.2\%$  versus  $52.0 \pm 19.0\%$ ,  $P = 0.06$ ). Selective endothelin-B receptor blockade reduced FBF ( $-11.0 \pm 3.9\%$ ,  $P = 0.02$ ) with no difference between placebo and cerivastatin therapy ( $-11.0 \pm 3.9\%$  versus  $-13.0 \pm 3.6\%$ ,  $P = 0.9$ ). Combined endothelin-A/endothelin-B receptor blockade increased FBF ( $39.8 \pm 13.4\%$ ,  $P < 0.01$ ) with no difference between placebo and cerivastatin therapy ( $39.8 \pm 13.4\%$  versus  $42.4 \pm 19.0\%$ ,  $P = 0.7$ ). There was a trend towards a reduction in the augmentation index between cerivastatin and placebo ( $6.2 \pm 2.7$  versus  $9.1 \pm 2.4$ ,  $n = 5$ ,  $P = 0.4$ ) compared with baseline ( $7.2 \pm 1.0$ ). In conclusion, statin therapy may decrease large artery stiffness and increase the vasodilating effects of endothelin-A receptor blockade.

**Key Words:** endothelin receptor blockade, statin, cerivastatin

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Endothelin-1 (ET-1) causes vasoconstriction via the Endothelin-A ( $\text{ET}_A$ ) and endothelin-B ( $\text{ET}_B$ ) receptors located on vascular smooth muscle cells, and vasodilatation via the  $\text{ET}_B$  receptor on endothelial cells, through a prostanoïd and nitric oxide (NO)-mediated effect. Thus,  $\text{ET}_A$  receptor blockade causes vasodilatation while the effects of  $\text{ET}_B$  receptor blockade will depend on the balance between vasodilating and vasoconstricting effects at different sites.

In healthy subjects, brachial artery administration of BQ-123, a selective  $\text{ET}_A$  antagonist, results in a forearm vasodilatation of  $\sim 40\%$ ,<sup>1–3</sup> whereas administration of BQ-788, a selective  $\text{ET}_B$  antagonist, causes a modest vasoconstriction of  $\sim 10\%$ .<sup>3</sup> If the mechanism of the vasodilatation is NO mediated, then in an NO-deficient state the dilatation to ET-1 at the endothelial  $\text{ET}_B$  receptor may be reduced, resulting in less vasoconstriction to BQ-788 and less vasodilatation to BQ123. Many studies have previously demonstrated evidence of endothelial dysfunction and an NO-deficient state in patients with hypercholesterolaemia.<sup>4–6</sup> Hypercholesterolaemia is also associated with a reduction in vascular compliance and a reduced response to vasoactive therapies such as nitrates. Some of these responses can be improved following lipid-lowering therapy.<sup>7,8</sup> In addition, it has been clearly demonstrated that reduction of plasma cholesterol reduces mortality in several patient groups.<sup>9–11</sup>

The aim of this study was to examine whether treatment of hypercholesterolaemia with an HMG CoA reductase inhibitor (cerivastatin) could increase vascular compliance and improve vasodilator responses to endothelin receptor antagonists.

## MATERIALS AND METHODS

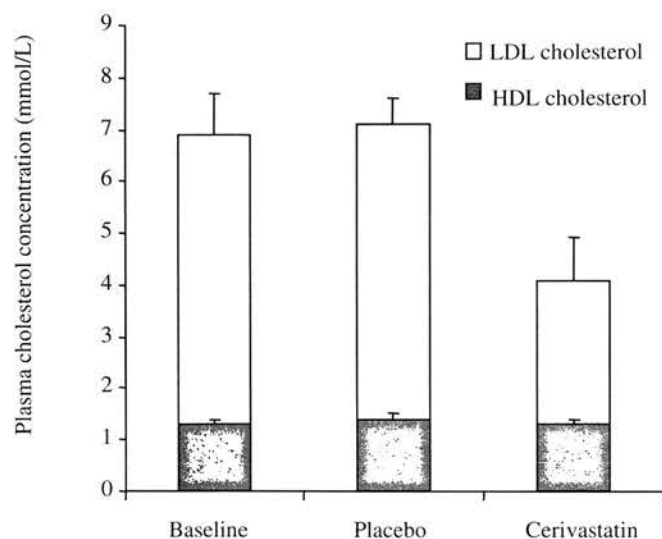
Five non-smoking, otherwise healthy, hypercholesterolaemic subjects were studied. Subjects were rejected if they had diabetes mellitus, renal impairment (creatinine, men  $> 180 \mu\text{mol/L}$ , women  $> 160 \mu\text{mol/L}$ ), a 10-year coronary heart disease risk  $> 30\%$ , a history of severe allergic reaction, a systolic blood pressure  $> 180$  mmHg or  $< 90$  mmHg, or were taking any regular medications. The study was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. Written informed consent was obtained from each subject before entry into the study. Subjects attended fasted for each study visit, which was performed in a quiet, draught-free, temperature-controlled room ( $22\text{--}24^\circ\text{C}$ ).

The techniques of venous occlusion plethysmography to measure forearm blood flow<sup>12</sup> and pulse wave analysis to measure the augmentation index and arterial stiffness<sup>13</sup> have been described in detail elsewhere. Subjects received, in a randomized, double-blind, cross-over manner, 8 weeks of treatment with either sucrose placebo or 800  $\mu\text{g}$  cerivastatin nocte (Bayer Plc, Newbury, U.K.). BQ123 and BQ788

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**FIGURE 1.** Plasma concentrations of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) after 8 weeks of treatment with placebo or cerivastatin.

(Clinalfa AG, Laufelfingen, Switzerland) were diluted in 0.9% saline (Baxter Healthcare Ltd, Thetford, U.K.) and infused via an intra-arterial needle over 80 minutes. Subjects received BQ123 (10 mol/minute), BQ-788 (1 mmol/minute) or both in a random order by 80-minute intra-arterial infusion at weeks 6, 7 and 8 and at weeks 14, 15 and 16. The order of intra-arterial drug infusion at the end of each treatment period was randomized and kept the same within patients (i.e. the order of endothelin antagonist forearm studies was consistent between treatment periods). Pulse wave analysis was performed and venous blood samples taken at weeks 0,

8 and 16. Data were examined by Student's *t* test (Excel v5.0; Microsoft). Results are expressed as the mean  $\pm$  standard error of the mean. Forearm blood flow and pulse wave analysis results are expressed as the percentage change from baseline. Statistical significance was taken at the 5% level.

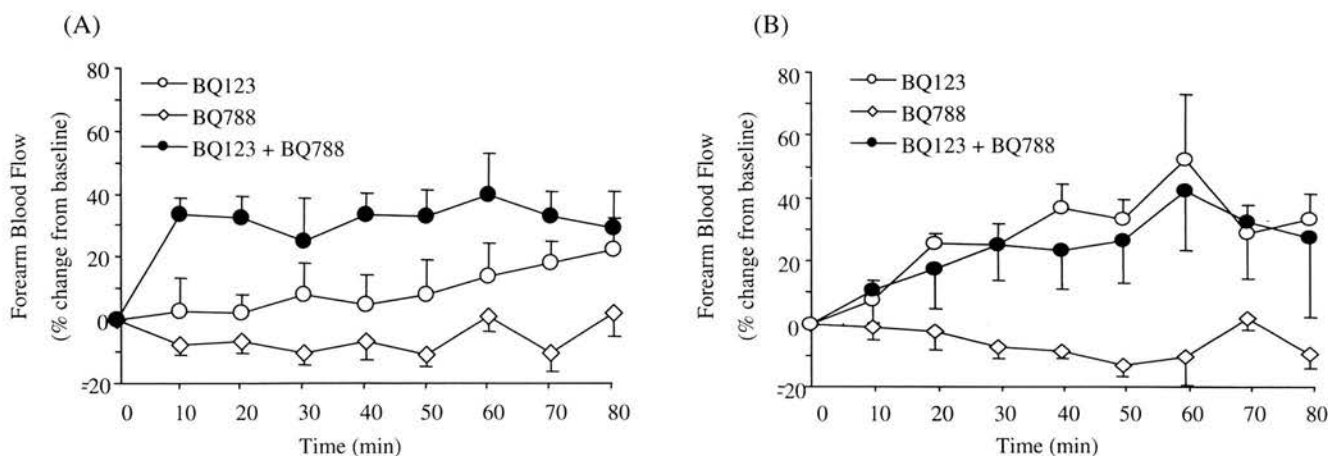
## RESULTS

Cerivastatin therapy for 8 weeks resulted in a reduction in total plasma cholesterol compared with baseline of 27% ( $5.4 \pm 0.4$  mmol/L versus  $7.3 \pm 0.4$  mmol/L,  $P = 0.04$ ); this was achieved by a reduction in low-density lipoprotein cholesterol (Fig. 1). There was no effect after 8 weeks of placebo treatment ( $7.4 \pm 0.4$  mmol/L versus  $7.3 \pm 0.4$  mmol/L,  $P = 0.9$ ). Selective  $ET_A$  receptor blockade caused an increase in forearm blood flow (FBF) ( $18.0 \pm 7.2\%$ ,  $P = 0.04$ ). Compared with placebo, 8 weeks of cerivastatin therapy caused a trend towards a further increase in FBF ( $18.0 \pm 7.2\%$  versus  $52.0 \pm 19.0\%$ ,  $P = 0.06$ ) (Fig. 2). Selective  $ET_B$  receptor blockade reduced FBF ( $-11.0 \pm 3.9\%$ ,  $P = 0.02$ ) with no difference between placebo and cerivastatin therapy ( $-11.0 \pm 3.9\%$  versus  $-13.0 \pm 3.6\%$ ,  $P = 0.9$ ). Combined  $ET_A/ET_B$  receptor blockade increased FBF ( $39.8 \pm 13.4\%$ ,  $P < 0.01$ ) with no difference between placebo and cerivastatin therapy ( $39.8 \pm 13.4\%$  versus  $42.4 \pm 19.0\%$ ,  $P = 0.7$ ). There was a trend towards a reduction in augmentation index between cerivastatin and placebo ( $6.2 \pm 2.7$  versus  $9.1 \pm 2.4$ ,  $n = 5$ ,  $P = 0.4$ ) compared with baseline ( $7.2 \pm 1.0$ ).

## DISCUSSION

This study has demonstrated that 8 weeks of statin therapy caused a trend towards increased vasodilatory responses to selective  $ET_A$  receptor blockade and a reduction

i n



**FIGURE 2.** Change in forearm blood flow from baseline with intra-arterial infusion of BQ123 (open circles), BQ788 (open diamonds) or the combination of BQ123 and BQ788 (closed circles) following 8 weeks of treatment with (A) placebo or (B) cerivastatin.

arterial stiffness. There were no differences in responses to selective ET<sub>B</sub> receptor or combined ET<sub>A</sub>/ET<sub>B</sub> receptor blockade between the placebo and statin treatment periods.

Consistent with studies in healthy volunteers, selective ET<sub>A</sub> receptor blockade with BQ-123 increased the FBF. However, there was a trend towards an increase in this vasodilatation after treatment with cerivastatin. These preliminary results are of interest, and there are several possible explanations for them. If the balance of effect at the ET<sub>B</sub> receptor is indeed vasodilatation, then the vasodilatation seen with ET<sub>A</sub> receptor blockade may have two components: first, the reduction in ET-1 tone mediated through the ET<sub>A</sub> receptor when blocked and, second, an increase in ET<sub>B</sub> receptor-mediated vasodilatation due to increased availability of ET-1 due to ET-1 displacement. The trend towards an increase in ET<sub>A</sub>-mediated vasodilatation following cerivastatin would support the hypothesis that statin therapy increases NO-mediated vasodilatation via the ET<sub>B</sub> receptor, given that there is no difference between groups when the ET<sub>B</sub> receptor is concomitantly blocked by combined BQ123 and BQ788 infusion. It has been previously shown that hypercholesterolaemia causes an impairment of NO-mediated vasodilatation and that this can be at least partially reversed by lipid-lowering therapy.<sup>8</sup> However, there may also be structural changes to the arterial wall that would be less likely to be reversed after 8 weeks of lipid lowering, and therefore a longer treatment period may be expected to have greater effects. Unfortunately this study was stopped prematurely as cerivastatin was withdrawn from the market due to concerns over interaction with fibrates. Despite this, the potential interaction between endothelin antagonists and statin therapy is of interest and deserves further investigation.

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# Endothelin-1<sub>[1-31]</sub> is Not Elevated in Men with Chronic Heart Failure

Stephen J. Leslie, Neil Johnston, Fiona E. Strachan, Alan Bagnall, Gillian A. Gray, David E. Newby, Martin A. Denvir, and David J. Webb

**Abstract:** Endothelin-1<sub>[1-31]</sub> is a recently discovered member of the endothelin family with vasoactive properties in several animal models and in man *in vivo*. It is generated from big endothelin-1 by human mast cell chymase and may be a novel intermediary peptide in the production of endothelin-1<sub>[1-21]</sub>. Given that both big endothelin-1<sub>[1-38]</sub> and chymase activity are increased in chronic heart failure, the aim of this study was to determine whether plasma endothelin-1<sub>[1-31]</sub> concentrations are elevated in patients with chronic heart failure. Plasma endothelin-1<sub>[1-31]</sub> concentrations were measured by enzyme-linked immunosorbent assay in nine patients with chronic heart failure, and nine age- and sex-matched control subjects. Consistent with previous studies, plasma concentrations of big endothelin-1<sub>[1-38]</sub> were elevated in patients compared with controls ( $17.1 \pm 4.4$  pg/mL vs  $8.9 \pm 3.4$  pg/mL,  $P = 0.002$ ), although there were no differences in plasma endothelin-1<sub>[1-21]</sub> ( $3.3 \pm 0.4$  pg/mL vs  $3.4 \pm 0.7$  pg/mL,  $P = 0.7$ ) or endothelin-1<sub>[1-31]</sub> (both  $1.1 \pm 0.1$  pg/mL,  $P = 0.2$ ) concentrations. We have demonstrated that patients with chronic heart failure have normal plasma endothelin-1<sub>[1-31]</sub> concentrations. This suggests that, in contrast to big endothelin-1<sub>[1-38]</sub>, plasma endothelin-1<sub>[1-31]</sub> is unlikely to be a useful prognostic marker in patients with chronic heart failure.

**Key Words:** endothelin-1<sub>[1-31]</sub> (ET-1<sub>[1-31]</sub>), chronic heart failure (CHF), assay, neuroendocrine

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New derivatives of endothelin have recently been characterized in humans. In contrast to the generation of endothelin-1 (ET-1<sub>[1-21]</sub>) by cleavage of the inactive precursor big endothelin-1 (big ET-1<sub>[1-38]</sub>), ET-1<sub>[1-31]</sub> is generated following the cleavage of big ET-1<sub>[1-38]</sub> at the Tyr<sup>31</sup>-Gly<sup>32</sup> bond by human chymase.<sup>1</sup> This endothelin (ET) intermediary peptide may be of clinical importance in conditions such as chronic heart failure (CHF) where there is increased generation of big ET-1<sub>[1-38]</sub> and increased chymase

activity in tissues including the heart<sup>2</sup> and blood vessels.<sup>3</sup> In addition, there is an increased amount of mast cells and human mast cell chymase at the shoulder regions of unstable coronary artery plaques,<sup>4,5</sup> and ET-1<sub>[1-31]</sub> may also contribute to the pathogenesis of ischemic heart disease and acute coronary syndromes.

In-vitro studies have shown that ET-1<sub>[1-31]</sub> is a vasoconstrictor in isolated porcine coronary arteries,<sup>6</sup> rabbit pulmonary arteries<sup>7</sup> and monkey trachea.<sup>8</sup> Endothelin-1<sub>[1-31]</sub> increases intracellular calcium in cultured human coronary artery smooth muscle cells<sup>9,10</sup> and human mesangial cells.<sup>11</sup> In addition, ET-1<sub>[1-31]</sub> causes vasoconstriction in human umbilical arteries<sup>8</sup> and we have previously reported that ET-1<sub>[1-31]</sub> is a vasoconstrictor in the human skin micro-circulation *in vivo*.<sup>12</sup> It is likely that ET-1<sub>[1-31]</sub> causes vasoconstriction via endothelin-A (ET<sub>A</sub>), and perhaps the endothelin-B (ET<sub>B</sub>), receptors either directly or following conversion to ET-1<sub>[1-21]</sub>.

Recently, a new sensitive and selective enzyme-linked immunosorbent assay (ELISA) has been developed to detect ET-1<sub>[1-31]</sub>.<sup>13</sup> The aims of this study were, therefore, to validate the measurement of plasma ET-1<sub>[1-31]</sub> using this ELISA, and to quantify circulating plasma ET-1<sub>[1-31]</sub> concentrations in patients with CHF and in healthy matched controls.

## METHODS

### Volunteer Selection

Nine male patients with moderate to severe chronic left ventricular dysfunction who had been stable on therapy for at least 3 months were recruited. They had a left ventricular ejection fraction of < 35%, shortening fraction of < 20% or left ventricular end diastolic diameter > 5.6 cm. Patients were compared with age- and sex-matched healthy controls. The study was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. Written informed consent was obtained from each subject before entry into the study.

### Blood Sampling and Plasma Extraction

All subjects omitted their medications on the day of study and attended after an overnight fast. Venous blood samples were collected into ethylenediaminetetraacetic acid

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(Sarstedt AG, Numbrecht, Germany), placed on ice and plasma was immediately separated by centrifugation (2500 g for 20 minutes at 4°C). Plasma samples were stored at -80°C until analysis. Plasma was acidified with an equal volume of 20% acetic acid before being extracted using methanol-activated Bond Elut C18 columns (Varian, Harbor City, CA, USA). The columns were perfused with deionized water and 10% acetic acid prior to the addition of plasma samples. The columns were then washed with 10% acetic acid and ethyl acetate, and the bound ET was eluted with 80% methanol:20% ammonium bicarbonate. The eluate was dried down under a continuous stream of nitrogen at 37°C and the dried eluates were reconstituted with assay buffer. Recovery fraction was estimated by adding known quantities of ET-1<sub>[1-31]</sub> to plasma.

## Peptides

ET-1<sub>[1-31]</sub> (Peptide Institute Inc., Osaka, Japan), big ET-1<sub>[1-38]</sub> (Clinalfa, Läufelfingen, Switzerland) and ET-1<sub>[1-21]</sub> (Clinalfa) were dissolved in physiological saline (0.9% Baxter Healthcare Ltd, Thetford, U.K.) and used to assess assay cross-reactivity.

## Assays

The assay for plasma big ET-1<sub>[1-38]</sub> and ET-1<sub>[1-21]</sub> has been previously described with intra-assay coefficients of variability of 7.0% and 7.2% respectively and inter-assay coefficients of variability of 9.0% and 9.3% respectively.<sup>14</sup> A commercially available solid phase sandwich ELISA for ET-1<sub>[1-31]</sub> (Immuno-Biological Laboratories Co. Ltd, Tokyo, Japan) was used. This technique was performed on reconstituted samples by using anti-human ET-1<sub>[25-31]</sub> rabbit immunoglobulin G. Briefly, 100 µL of sample extract, standard or quality control as well as 100 µL of ET-1<sub>[1-31]</sub> antibody, was incubated for 24 hours at 4°C. Following incubation, the wells were vigorously washed with buffer, 100 µL of labelled antibody was added, followed by incubation for a further 30 minutes at 37°C. After further washing, tetramethylbenzidine buffer was added, and incubated for 30 minutes in the dark. Finally, 100 µL of 2 M sulfuric acid was added and the wells were read using an automated plate reader at 450 nm.

## Data Handling and Statistical Analysis

Cross-reactivity was determined by calculating area under the curves for big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub>, and ET-1<sub>[1-21]</sub>, and was expressed as a percentage of area under the curve for ET-1<sub>[1-31]</sub>. Intra-assay coefficients of variation were calculated from mean values divided by standard deviation and expressed as a percentage. Plasma concentrations are expressed as mean ± standard error of the mean. Data were examined by analysis of variance (ANOVA; Excel® v5.0, Microsoft). Statistical significance was taken at the 5% level.

## RESULTS

Patient details are shown in Table 1.

### Recovery of ET-1<sub>[1-31]</sub>

Recovery of ET-1<sub>[1-31]</sub> during extraction was 73%. This is comparable to the extraction efficiency for big ET-1 and ET-1.<sup>14</sup>

### Specificity of ELISA for ET-1<sub>[1-31]</sub>

Standard curves for big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub>, and ET-1<sub>[1-21]</sub> were constructed (Fig. 1). The cross-reactivities for the assay were 3.8% for big ET-1<sub>[1-38]</sub>, 100% for ET-1<sub>[1-31]</sub>, and 0% for ET-1<sub>[1-21]</sub>. Intra-assay coefficients of variation were 14.7%, 8.8% and 6.7% at plasma ET-1<sub>[1-31]</sub> concentrations of 10 pg/mL, 20 pg/mL and 40 pg/mL, respectively.

### Plasma Concentrations of Big ET-1, ET-1 and ET-1<sub>[1-31]</sub>

Plasma big ET-1<sub>[1-38]</sub> concentrations were elevated in patients compared with controls (17.1 ± 4.4 vs 8.9 ± 3.4 pg/mL, *P* = 0.002). However, there were no differences in the plasma concentrations of ET-1<sub>[1-21]</sub> (3.3 ± 0.4 vs 3.4 ± 0.7 pg/mL, *P* = 0.7) or ET-1<sub>[1-31]</sub> (both 1.1 ± 0.1 pg/mL, *P* = 0.2) (Fig. 2).

## DISCUSSION

This study confirms that a new ELISA has high specificity for ET-1<sub>[1-31]</sub>, with low cross-reactivity for both ET-1<sub>[1-21]</sub> and big ET-1<sub>[1-38]</sub>. In addition, we have demonstrated, for the first time, that ET-1<sub>[1-31]</sub> immunoreactivity is present in human plasma of both CHF patients and healthy subjects. Despite elevation of the precursor peptide big ET-1<sub>[1-38]</sub>, plasma ET-1<sub>[1-31]</sub> concentrations were normal in patients with CHF.

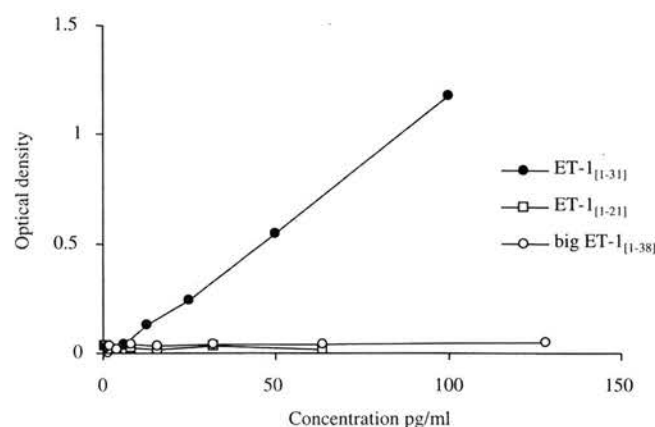
We have demonstrated that the commercially available assay for ET-1<sub>[1-31]</sub> has a high degree of specificity for this form of endothelin compared with big ET-1<sub>[1-38]</sub> and ET-1<sub>[1-21]</sub>. The ELISA utilizes an antibody that recognizes the 7 amino acid carboxy terminus of ET-1<sub>[1-31]</sub>. Given that big ET-1<sub>[1-38]</sub> also shares this common sequence, it may have been anticipated that significant cross-reactivity might occur. However, we report a very low level of cross-reactivity and this is likely to reflect differences in immunoreactivity conferred by the tertiary structure of big ET-1<sub>[1-38]</sub> and ET-1<sub>[1-31]</sub>.

Plasma big ET-1 concentrations are elevated in patients with CHF and correlate with disease severity.<sup>15</sup> Our findings are consistent with these earlier data, although plasma ET-1<sub>[1-21]</sub> concentrations were not elevated. This was unexpected as plasma ET-1<sub>[1-21]</sub> concentrations are also reported to be elevated in patients with CHF.<sup>15</sup> However, big ET-1<sub>[1-38]</sub> plasma concentrations are a more reliable marker of CHF and, in our study, these were increased by

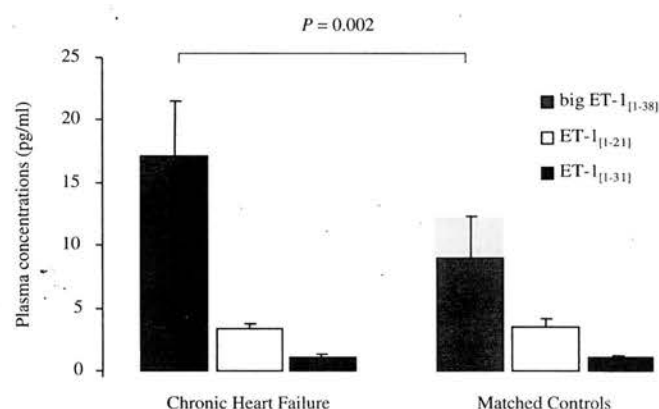
**TABLE 1.** Test results for patients and age- and sex-matched controls

	Age (years)	BMI (kg m <sup>-2</sup> )	Chol (mmol/L)	Smoker	BP (mmHg)	NYHA	Drugs
Patient							
1	67	22	6.9	N	143/105	III	ASA, EN, BU, AMIO
2	78	26	6.4	N	195/95	II	DIS, BU
3	57	28	4.2	N	116/68	III	ASA, EN, FRU, SIM, DIG, ISMO
4	47	26	6.1	Y	112/72	II	ASA, EN, FRU, ISMO
5	69	29	6.1	N	121/68	II	ASA, EN, FRUS, BIS
6	76	25	5.3	N	140/80	III	ASA, EN, FRU
7	80	26	5.0	Y	190/90	II	ASA, LOS, FRU, DIG
8	77	28	4.2	Y	175/80	III	ASA, CAP, AMIO
9	81	23	5.5	N	120/60	II	ASA, EN, DIG, SIM
Average	70	26	5.5		146/80		
SEM	4	1	0.3		11/5		
Control							
1	67	26	4.8	N	124/80	NA	None
2	77	29	5.8	N	158/90	NA	None
3	73	24	4.3	N	132/88	NA	None
4	48	25	5.1	N	128/80	NA	None
5	67	26	4.7	N	145/87	NA	None
6	73	23	7.0	N	135/72	NA	None
7	81	27	5.4	N	127/64	NA	None
8	77	27	6.7	N	117/77	NA	None
9	79	29	4.4	N	103/72	NA	None
Average	71	26	5.4		130/79		
SEM	3	1	0.3		5/3		

BMI, body mass index; Chol, serum cholesterol; BP, blood pressure; NYHA, New York Heart Association; ASA, aspirin; EN, enalapril; BU, bumetanide; AMIO, amiodarone; DIS, dipyridamole; FRU, frusemide; SIM, simvastatin; DIG, digoxin; ISMO, isosorbide mononitrate; BIS, bisoprolol; LOS, losartan; CAP, captopril.

**FIGURE 1.** Standard curves for big endothelin-1 (ET-1)<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub> and ET-1<sub>[1-21]</sub>.

approximately twofold. We have also recruited stable patients who were well controlled on medical therapy. It is likely, therefore, that our patient population had less neurohormonal activation and may have had less generation of big ET-1<sub>[1-38]</sub> than those enrolled into previous studies.

**FIGURE 2.** Concentrations of plasma big endothelin-1 (ET-1)<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub> and ET-1<sub>[1-21]</sub> in patients with chronic heart failure and matched controls.

In patients with CHF, there does not appear to be a general increase in generation of ET-1<sub>[1-31]</sub>. This study does not however exclude the possibility of local increases in ET-1<sub>[1-31]</sub> production, such as within areas of inflammation. Moreover, local ET-1<sub>[1-31]</sub> generation may be specific to

those tissues with increased chymase activity such as failing human myocardium.<sup>2</sup> In addition, increased chymase activity is seen in mast cells at the shoulder regions of coronary atheromatous plaques<sup>4,5</sup> and ET-1<sub>[1-31]</sub> may be implicated in the etiology of coronary artery spasm at the time of plaque rupture. Further studies are clearly required to determine how ET-1<sub>[1-31]</sub> is processed and to identify situations where increased local ET-1<sub>[1-31]</sub> generation may occur.

In conclusion, this study has shown that plasma ET-1<sub>[1-31]</sub> concentrations can be measured reliably but, in contrast to big ET-1<sub>[1-38]</sub>, do not appear to be elevated in patients with heart failure. These data suggest that ET-1<sub>[1-31]</sub> is not useful as a predictive test and that generation is not sufficient to increase circulation plasma concentrations in patients with stable CHF.

### ACKNOWLEDGMENTS

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# Non-invasive measurement of cardiac output in patients with chronic heart failure

Stephen J. Leslie, Sinéad McKee, David E. Newby, David J. Webb and Martin A. Denvir

**Objectives** The measurement of cardiac output by thoracic bioimpedance has been previously assessed in several studies. However, there continues to be disagreement as to whether this technique is sufficiently accurate for use in clinical practice or research. The current study aimed to compare thoracic bioimpedance ( $CO_{TB}$ ) with thermodilution ( $CO_{TD}$ ) in patients with stable chronic heart failure.

**Methods and results** A total of 282 paired measurements of cardiac output from 11 patients were analysed. There was good correlation between  $CO_{TB}$  and  $CO_{TD}$  ( $r=0.76$ ,  $P<0.0001$ ). However, Bland-Altman analysis revealed an average difference between values of  $0.3 (2.2) \text{ l/min}$  ( $P=0.02$ ), suggesting a small average bias but marked variability in results. There was no significant correlation when results were expressed as percentage change from baseline and a significant average difference between values of  $10.1 (30.1)\%$ . There was no difference in between-day repeatability between thermodilution and thoracic bioimpedance [ $-0.2 (1.2)$  versus  $0.1 (1.0) \text{ l/min}$ ,  $P=0.7$ ].

**Conclusions** This study demonstrates a correlation between the techniques but shows a poor level of agreement. The method of  $CO_{TB}$  underestimated cardiac output compared with  $CO_{TD}$ , and this difference appeared greater with higher cardiac outputs. Agreement was worse when results were expressed as change from baseline. The present study does not support the use of thoracic bioimpedance in its current form as an alternative to thermodilution in stable patients with chronic heart failure. *Blood Press Monit* 9:277-280 © 2004 Lippincott Williams & Wilkins.

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**Keywords:** thoracic bioimpedance, heart failure, haemodynamics

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## Introduction

The most popular method for measurement of cardiac output is the thermodilution technique although it may not be suitable for use in certain situations, such as volunteer research studies, because of its invasive nature. In contrast, thoracic bioimpedance is a relatively simple, non-invasive technique for measuring cardiac output, which has been employed in several clinical studies. However, there continues to be disagreement as to whether this technique is sufficiently accurate and precise to be of use in clinical practice or research.

Many studies have supported the use of thoracic bioimpedance [1-6], while others suggest it is not sufficiently accurate [7-9]. The applicability of thoracic bioimpedance has been assessed in several groups of unstable patients such as those following cardiac surgery [3], in acute heart failure [1] and in critically ill patients on intensive care units [3]. In addition, some workers have based assessment of clinical applicability on single point measurements rather than trends [4,8]. This may limit their applicability since thoracic bioimpedance may

be more accurate at measuring changes in cardiac output rather than absolute values [2]. There are few studies comparing thoracic bioimpedance with thermodilution for determining absolute, as well as relative changes in cardiac output in stable patients with chronic heart failure. The aim of this study was to compare the techniques of thoracic bioimpedance and thermodilution for the measurement of cardiac output in patients with stable chronic heart failure, during an acute study with a pharmacological intervention.

## Methods

### Patient selection

Eleven patients with chronic heart failure (New York Heart Association Grade II/III) due to left ventricular dysfunction attended for a total of 30 visits (maximum three visits per patient). All patients had an ejection fraction  $\leq 35\%$  (by echocardiography using the biplanar Simpson's rule) and were stable on treatment for at least three months. All patients were in sinus rhythm and none had a cardiac pacemaker. The study was undertaken with the approval of the local research ethics committee and in

accordance with the Declaration of Helsinki. Written informed consent was obtained from each subject before entry into the study.

## Measurements

### Thermodilution

Cardiac output was measured by the thermodilution technique using the semi-continuous single multi-lumen thermodilution cardiac output pulmonary artery catheter (Swan-Ganz CCOMBO-CCO/SVO2; Edwards Lifesciences LLC, Irvine, California, USA) [10,11]. This device has a thermal filament which transfers a safe level of heat to the surrounding blood in a pulsed, on-off manner every 30 s. Changes in the temperature of the blood distal to this are measured by a distal rapid-response thermistor in the pulmonary artery. The cardiac output is then calculated automatically using a modified Stewart-Hamilton equation [12].

### Thoracic bioimpedance

Thoracic bioimpedance was performed using the BioMed<sup>®</sup> NCCOM3 system (BioMed Medical Manufacturer Ltd., Irvine, California, USA). Four pairs of low impedance electrodes are used, two on either side of the thorax at the level of the xiphoid sternum in the mid axillary line and two on the lateral aspect of the base of the neck. In each pair, the upper electrode delivers a small current that is sensed by the lower electrode, thus impedance is measured and the cardiac output can be calculated.

### Protocol

Patients were asked to attend after an overnight fast and to omit their regular medications on the morning of the study. The studies were conducted in a quiet room maintained at a constant temperature (22–24°C). A pulmonary artery catheter was inserted, via a 9F femoral venous sheath, into the right pulmonary artery. Before starting the drug infusion, patients underwent an equilibration period of >90 min until blood pressure, heart rate and cardiac output were stable, with three consecutive measurements within 10%. Systemic concentrations of endothelin antagonists were administered intravenously at low dose then high dose over 15 min, 1 h apart. Endothelin blockade causes vasodilatation and in patients with heart failure an increase in cardiac output. These results have been described elsewhere [13].

### Data analysis and statistics

Near simultaneous measurements of CO were obtained from the mean of three consecutive recordings at each period for both CO<sub>TD</sub> and CO<sub>TB</sub>. Data are expressed as mean value (standard error of the mean; SEM). Percentage changes in cardiac output from baseline were also studied. Within device repeatability was assessed by analysing baseline measurements of cardiac output in the same patient on difference days. The method of Bland

and Altman was used to describe the relationship between CO<sub>TD</sub> and CO<sub>TB</sub>. Bland-Altman analysis can be used to evaluate two techniques where the mean of the differences between two values is a measure of accuracy (or bias) and the standard deviation of these values gives a measure of precision [14]. Statistical analysis was performed using Student's *t*-test repeatability. Statistical significance was taken at the 5% level.

## Results

There were no adverse events and the study was well tolerated by all patients. Ten (91%) of the patients were male, and three (27%) had a NYHA score of II with the remaining eight (73%) having a score of III. The other characteristics of the patients are presented in Table 1. The results of the haemodynamic effects of the study have been reported elsewhere [13]. In brief, administration of systemic doses of endothelin antagonist resulted in peripheral vasodilation and an increase in cardiac output by a maximum of approximately 30%. The use of both selective and non-selective endothelin blockade at low and high dose allowed for a range of changes in cardiac output to be investigated.

### Comparison between thoracic bioimpedance and thermodilution

There was good correlation between CO<sub>TB</sub> and CO<sub>TD</sub> ( $r = 0.76$ ,  $P < 0.0001$ ) although Bland-Altman analysis revealed an average difference between values of 0.3 (2.2) l/min ( $P = 0.02$ ), suggesting a small average bias but marked variability in results (Fig. 1). There was no significant correlation when results were expressed as percentage change from baseline and a significant average difference between values of 10.1 (30.1)% (Fig. 2).

### Repeatability of baseline measurements

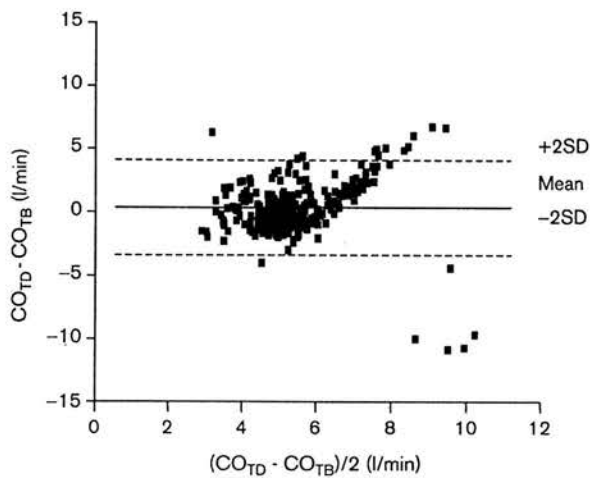
Within device repeatability was assessed by analysing baseline measurements of cardiac output in the same patient on difference days. There was no difference in

Table 1 Baseline patient characteristics

Parameter	Mean (SD)
Age (years)	61 ± 9
BMI (kg/m <sup>2</sup> )	27 ± 2
HR (bpm)	63 ± 9
SBP (mmHg)	136 ± 21
DBP (mmHg)	77 ± 12
CVP (mmHg)	5 ± 1
MPAP (mmHg)	16 ± 2
CO (l/min)	5.3 ± 0.7
CI (l/min per m <sup>2</sup> )	2.6 ± 0.2
SVR (dynes.s.cm <sup>-5</sup> )	1358 ± 274
PVR (dynes.s.cm <sup>-5</sup> )	84 ± 25
PCWP (mmHg)	11 ± 3

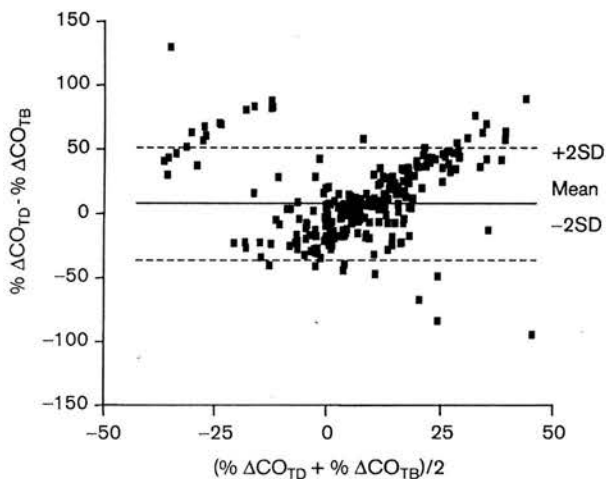
Body mass index (BMI), heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP), central venous pressure (CVP), mean pulmonary artery pressure (MPAP), cardiac output (CO) by thermodilution, systemic vascular resistance (SVR), pulmonary vascular resistance (PVR), pulmonary capillary wedge pressure (PCWP) (Invasive measurements made by thermodilution technique)

Fig. 1



Bland-Altman analysis of absolute cardiac output by thermodilution  $CO_{TD}$  and thoracic bioimpedance  $CO_{TB}$ .

Fig. 2



Bland-Altman analysis of percentage change in cardiac output from baseline by thermodilution  $CO_{TD}$  and thoracic bioimpedance  $CO_{TB}$ . (Note difference in calibration between the axes).

between-day repeatability between thermodilution and thoracic bioimpedance  $[-0.2 (1.2)$  versus  $0.1 (1.0)$  l/min,  $P = 0.7$ ].

## Discussion

There is no 'perfect' method for measuring cardiac output although the technique of thermodilution is most commonly used. However, due to the invasive nature of pulmonary artery catheterization there is considerable

interest in the non-invasive technique of thoracic bioimpedance. Thoracic bioimpedance has the advantage that it is relatively inexpensive and easy to use. However, its accuracy has been questioned.

This study has compared the techniques of thoracic bioimpedance against thermodilution in stable patients with chronic heart failure undergoing an acute haemodynamic intervention study. A meta-analysis of thoracic bioimpedance studies found a good correlation between thoracic bioimpedance and reference techniques ( $r = 0.66$ ) [15]. In keeping with these studies we have found a good correlation between thoracic bioimpedance and a thermodilution technique ( $r = 0.76$ ). However, while there is a positive correlation between the techniques, there is a poor level of agreement. The method of thoracic bioimpedance systematically underestimated cardiac output compared with thermodilution, and this difference appeared to be greater with higher cardiac output. Several studies have supported the use of thoracic bioimpedance when measuring trends in cardiac output [1-6]. However, the current study does not support this finding, with poor agreement between values when expressed as percentage change from baseline despite reasonable repeatability of baseline measurements.

We observed an unacceptably low level of agreement both for absolute and changes in cardiac output. Previous studies have assessed thoracic bioimpedance in critically ill patients or patients after mechanical ventilation or undergoing cardiac surgery. Pulmonary oedema and mechanical ventilation can affect thoracic bioimpedance and many previously studied patients may have been unsuitable for measurement of cardiac output by thoracic bioimpedance. The patients in this present study had stable chronic heart failure, were in sinus rhythm and in the controlled setting of an acute haemodynamic intervention study. It would have been expected that these optimal conditions would provide the best opportunity for thoracic bioimpedance to be comparable with thermodilution, given that patients were stable, self-ventilating and had no pulmonary oedema. However, the findings suggest that thoracic bioimpedance is not comparable to thermodilution in patients with stable chronic heart failure. Furthermore, invasive monitoring using pulmonary artery catheters may have additional advantages over non-invasive thermodilution in that they allow direct measurement of blood pressure and allow sampling of blood from the pulmonary artery.

Some of the differences noted in our study may be due to slight difference in the timing of cardiac output measurements, as thoracic bioimpedance cannot be measured at exactly the same time as thermodilution. However, measurements were within 60 s of each other

and therefore this is unlikely to explain the degree of poor agreement between these two techniques, given that overall changes in cardiac output throughout the study were slow to develop in response to intravenous infusion of the endothelin antagonist.

In conclusion, thoracic bioimpedance, in its current form, does not appear to sufficiently agree with invasive thermodilution techniques for measuring cardiac output in patients with stable chronic heart failure. Therefore, it should not be routinely used as an alternative to thermodilution in patients with heart failure even in the controlled setting of a clinical study.

### Acknowledgements

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## Comparison of two plethysmography systems in assessment of forearm blood flow

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**Leslie, Stephen J., Teresa Attinà, Ellen Hultsch, Luc Bolscher, Matthias Grossman, Martin A. Denvir, and David J. Webb.** Comparison of two plethysmography systems in assessment of forearm blood flow. *J Appl Physiol* 96: 1794–1799, 2004. First published January 29, 2004; 10.1152/jappphysiol.00567.2002.—Venous occlusion plethysmography is widely used to assess forearm blood flow (FBF). We compared the established Hokanson system (HEC4) with a newly developed Filtrass 2001 system (F2001). The HEC4 uses mercury-in-Silastic strain gauges, whereas F2001 detects volume changes with a nonmercury linear displacement device. The aim of this study was to evaluate the new F2001 against the HEC4 in terms of repeatability and systematic bias. Ten subjects were studied on 4 separate days in random order using either the HEC4 on both arms, the F2001 on both arms, the HEC4 on the right arm with the F2001 on the left, or the F2001 on the right arm and the HEC4 on the left. Stroop's colored word conflict test and postocclusive hyperemia were used to increase FBF, and lower body negative pressure was used to lower FBF. Stroop's colored word conflict test and lower body negative pressure increased ( $24.6 \pm 1.5\%$ ,  $n = 240$ ,  $P < 0.0001$ ) and decreased ( $18.7 \pm 0.8\%$ ,  $n = 240$ ,  $P < 0.0001$ ) FBF, respectively. Postocclusive hyperemia after occlusion times of 5, 8, and 13 min substantially increased FBF by  $390 \pm 86$ ,  $756 \pm 217$ , and  $851 \pm 132\%$ , respectively. Repeatability was not different between the devices ( $0.10 \pm 2.37$  vs.  $-0.47 \pm 1.92$  l/min,  $n = 125$ ,  $P > 0.05$ ), and there was no systematic bias. The F2001 is a newly developed plethysmography system that does not utilize mercury and is suitable for assessing changes of FBF in physiological studies.

validation; Stroop's colored word conflict test; lower body negative pressure

VENOUS OCCLUSION PLETHYSMOGRAPHY has been used to study forearm blood flow (FBF) for almost 100 years (4). The underlying principle is simple: by obstructing venous outflow, but not arterial inflow, the forearm volume initially increases in proportion to the FBF. The standard technique for the assessment of this change in FBF, using a circumferential mercury-in-Silastic strain gauge as part of a Wheatstone bridge to detect increases in forearm circumference and derive volume changes, has remained essentially unchanged for 50 years (24). Venous occlusion plethysmography is now a well-validated (19) and widely used tool to study mechanisms of human vascular control, particularly when coupled with brachial artery infusion to study effects of drugs and mediators on the forearm vasculature (12). In humans, this approach to vascular pharmacology has a distinct advantage over other techniques in that vessels are studied in their physiological environment (1,

12). A commonly used and well-validated device employing the above principles is the Hokanson EC4 system (HEC4) (5, 7, 10, 17, 20, 22).

Recently, a new device, the Filtrass 2001 system (F2001), has been developed, particularly to measure capillary permeability. However, this device may also be useful for measuring FBF. It is based on similar principles as HEC4 but with some important differences. With the F2001, forearm circumferential measurement is measured by displacement of a freely moveable core of a linear variable displacement transducer (LVDT) attached to a circumferential plastic monofilament (Fig. 1). A linear relationship exists over a large range of calibration movements (6). The system is mechanically and electrically calibrated to measure differences of  $10 \mu\text{m}$  and undergoes internal calibration for changes in temperature and tissue compliance at each time point. The potential utility of this device in forearm venous occlusion plethysmography has not previously been assessed.

The aim of this study was to compare the F2001 against the established HEC4 in terms of repeatability, systematic bias, and ease of use. To compare the F2001, subjects underwent simple, noninvasive systemic interventions to reduce or increase FBF. It was assumed that systemic hemodynamic changes would result in similar changes in FBF simultaneously in both arms (9). To assess higher blood flows, postocclusive hyperemia (POH) was used. In this study, the repeatability of each individual device could be assessed when the same device was used on both arms. Comparison of one system against the other on different arms allowed examination for systematic bias between devices.

### METHODS

**Subjects.** Ten healthy subjects (9 men) aged 22–30 yr were recruited for this study, which was undertaken in accordance with the Declaration of Helsinki and with the approval of the local research ethics committee. Written, informed consent was obtained from each subject. All subjects abstained from vasoactive drugs for at least 1 wk, from alcohol and cigarettes for 24 h, and from food and caffeine for at least 3 h before each study visit.

**Study design.** Volunteers attended for two separate study periods of four visits each. The protocol was similar on each of the first four study days except for the plethysmography device used. The devices were studied in random order using either the HEC4 on both arms, the F2001 on both arms, the HEC4 on the right arm with the F2001 on the left arm, or the F2001 on the right arm and the HEC4 on the left arm. During the second four study visits, POH was employed to cause a range of increases in FBF.

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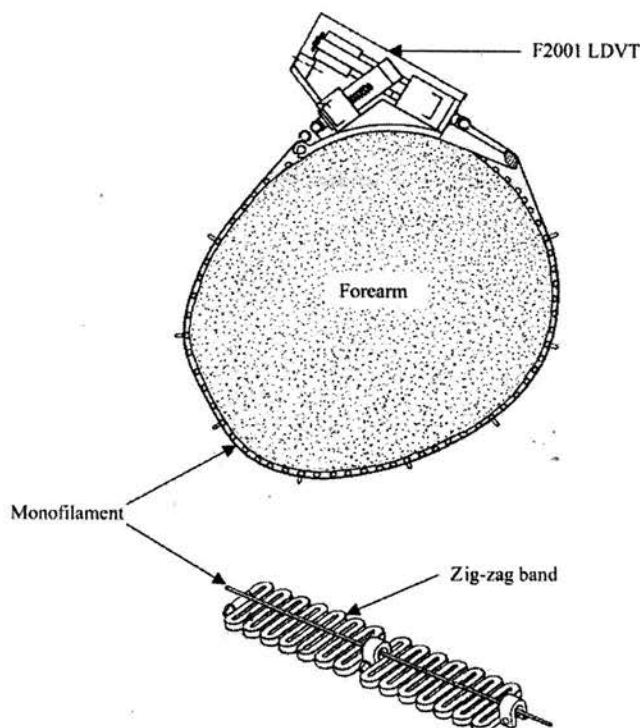


Fig. 1. Filtrass 2001 plethysmography device and Filtrass 2001 zigzag band and linear displacement device

**Study protocol.** The study visits were performed on different days in a quiet, draft-free, temperature-controlled room (22–24°C). During the low-flow experiments, the volunteer was subjected to either Stroop's colored word test (SCWT), lower body negative pressure (LBNP), or a rest period. Mean arterial pressure and heart rate were measured during minute 6 and FBF was measured during the last 3 min of each 10-min time period. During the high-flow experiments, volunteers were exposed to 5, 8, and 13 min of arterial occlusion resulting in postocclusive reactive hyperemia.

**Venous occlusion forearm plethysmography.** Both devices were employed in a similar manner using the standard methodology employed in our unit (25). In brief, venous occlusion was achieved by inflating cuffs around the upper arm to above venous but below arterial pressure (~40 mmHg). The arm was placed above the level of the right atrium, and the upper arm cuff was inflated for 10 s then deflated for 5 s. This cycle was repeated for the last 3 min of each 10-min study time period. Hand blood flow was predominantly through skin blood vessels rather than skeletal muscle and thus had different control mechanisms than FBF (21, 23, 24). Therefore, the hands were excluded from the circulation by inflating cuffs around the wrists to above systolic pressure (~200 mmHg) before FBF measurement. The strain gauges were placed around the forearm at the point of greatest circumference.

There were some features unique to each device. HEC4 (D. E. Hokanson, Bellevue, WA) consists of several discrete components: two HEC4 plethysmographs, two Hokanson E20 rapid cuff inflators, a Hokanson AG 101 cuff inflator air resource, strain gauges, and Hokanson wrist and upper arm cuffs. HEC4 uses electrically calibrated mercury-in-Silastic strain gauges, which were calibrated at the beginning of each study. HEC4 occupies a total volume of 1.4 m<sup>3</sup>.

The F2001 LVDT (DOMED Medizintechnik, Munich, Germany) was positioned on the forearm skin at a similar position to the mercury-in-Silastic strain gauges and fixed to the forearm with adhesive

tape. The plastic monofilament component surrounding the forearm was supported on the skin by a "zig-zag band" (Fig. 1). Before the start of measurements, the surface of the monofilament was coated with synthetic oil (DOMED Medizintechnik) to reduce friction and then inserted into the main body of the LVDT. The LVDT was calibrated at the beginning of each study, and the resting tension on the monofilament was adjusted automatically at each time point. The F2001 is a smaller, more compact device than the HEC4, with all components contained within a single housing with a volume of 0.22 m<sup>3</sup>.

**LBNP.** The lower body of the subject was placed in an airtight steel chamber and sealed with a pneumatic belt around the waist. Suction was applied by a low-pressure vacuum device, and the negative pressure within the chamber was kept constant by a servomechanical pressure regulator (Department of Medical Physics, Western General Hospital, Edinburgh, UK). LBNP (15 mmHg) was applied for 10 min to reduce FBF. LBNP caused venous pooling, which, via baroreceptor unloading and selective activation of elements of the sympathetic nervous system, results in an increase in systemic vascular resistance and forearm vascular resistance and thus a reduction in FBF. Previous work (13) has shown that there is no effect on mean arterial pressure or heart rate at this level of negative pressure.

**SCWT.** SCWT consists of several pages of the words "blue," "red," "yellow," and "green," each printed in different colors of ink (blue, red, yellow, and green) in a random order. The subject is asked to state the color of the ink in which the word is printed not the printed word. Mental conflict arises because the learned response is to read the word rather than report the color. This task was performed at a steady rate of ~100 words/min with a metronome and observer acting as a guide to encourage volunteers to keep to the pace. To induce reproducible mental stress in our subjects, we applied the SCWT for 10 min. Responses to the SCWT include a decrease in forearm vascular resistance and a marked increase in FBF (14). SCWT also causes an increase in mean arterial pressure, heart rate, and cardiac output and a fall in systemic vascular resistance. Increases in FBF are stable and reproducible after 9 min (8). However, forearm vasodilator responses to SCWT may become diminished with repeated exposure (11) and, to cause a range of FBF changes, three 10-min exposures to SCWT were performed at each study visit.

**POH.** Postocclusive hyperemia (POH) can cause marked increases in FBF (16). Brachial artery occlusion was applied by manually inflating an upper arm cuff to at least 60 mmHg above systolic pressure. POH of the forearm resulted after the occlusion was released. A range of occlusion times (5, 8, and 13 min) were used to assess the devices over a range of FBF.

**Data acquisition and statistical analysis.** FBF was obtained from the mean of the last five consecutive recordings of each period. Both systems allow the manual rejection of curves if rendered unsuitable for analysis by movement artifact. Each slope recording was taken from the steep linear part of the response curve. In the high-flow experiments, the plateau phase was reached more quickly, but the steep linear portion of the curve was still easily identified and assessed over three or more heartbeats. Data obtained from the Hokanson plethysmograph were stored on a Macintosh computer using the Chart version 3.3 software (MacLab, ADInstruments). Data were further analyzed offline using the Chart version 3.3 software and a template spreadsheet (Excel 5.0, Microsoft). Data from the F2001 were analyzed in a similar manner using a PC-based automatic analysis program, which fits a curve to the measured slope and produces a mean value for FBF.

There is no "gold standard" for the noninvasive measurement of FBF. Repeatability of the devices was quantified by the average difference between the changes in FBF from the preceding rest period, in response to stimuli, measured by the same device when used simultaneously on left and right arms (HEC4 on both arms or the F2001 on both arms). Therefore, assuming stimuli will affect the FBF in each arm to a similar degree, the closer to zero the difference in

FBF between each arm and the smaller the standard deviation of results, the more repeatable the device. Systematic bias between devices was determined by the average of the differences from the mean when different devices were simultaneously used to measure the changes in FBF (HEC4 on the right arm with the F2001 on the left arm or F2001 on the right arm and the HEC4 on the left arm) and presented as Bland-Altman plots (3).

The effects of the SCWT, LBNP, and POH on FBF were assessed using all data from both devices on left and right arms and expressed as mean percentage changes from baseline  $\pm$  SE. Mean differences were assessed by using Student's *t*-test. Statistical significance was taken at the 5% level. The study had 80% power to show a 7.5% difference between the devices, assuming a 15% standard deviation in FBF measurement.

## RESULTS

None of the subjects were obese. All were right handed with correspondingly larger right forearm circumferences ( $264 \pm 5$  vs.  $259 \pm 5$  mm,  $P < 0.01$ ). However, there was no difference between baseline blood flow between the left and right arms ( $3.1 \pm 0.2$  vs.  $2.6 \pm 0.1$  ml  $\cdot$  100 ml $^{-1}$   $\cdot$  min $^{-1}$ ,  $P = 0.1$ ). The HEC4 cuff is broader than the F2001 cuff and inflates in  $<1$  s, whereas the F2001 cuff takes 3–4 s to inflate. There did not appear to be any difference in cuff artifacts between the two systems as a result.

LBNP and SCWT caused the expected changes in FBF, which were repeatable within study visits (Fig. 2A). LBNP caused a decrease in FBF ( $-18.7 \pm 0.8\%$ ,  $n = 240$ ,  $P < 0.0001$ ), and SCWT caused an increase in FBF ( $24.6 \pm 1.5\%$ ,  $n = 240$ ,  $P < 0.0001$ ). Forearm hyperemia after occlusion times of 5, 8, and 13

min substantially increased FBF by  $390 \pm 86$ ,  $756 \pm 217$ , and  $851 \pm 132\%$ , respectively (all  $P < 0.0001$ ) (Fig. 2B).

**Comparison between HEC4 and F2001.** When used to measure changes in the left and right arm, HEC4 showed no difference in the absolute change in FBF between arms ( $0.10 \pm 2.37$  l/min,  $n = 125$ ,  $P > 0.05$ ). When used to measure simultaneous changes in the left and right arm, F2001 showed no difference in absolute changes in FBF between arms ( $-0.47 \pm 1.92$  l/min,  $n = 125$ ,  $P > 0.05$ ). There was no difference between HEC4 and F2001 ( $0.10 \pm 2.37$  vs.  $-0.47 \pm 1.92$  l/min,  $n = 125$ ,  $P > 0.05$ ). There was good correlation between the devices (Fig. 3). Bland-Altman plots reveal no systematic bias between the devices or in relation to flow values when used to measure simultaneous changes in response to the systemic stimuli described above (Fig. 4).

## DISCUSSION

FBF measurement is widely used in physiological and pharmacological studies in the clinical laboratory setting (12). Existing mercury-in-Silastic (24) and Dohn air-filled cuffs have been widely used and appropriately validated (18) for measuring baseline and relative changes in blood flow. The present study was designed to assess repeatability and systematic bias using a new device for measuring FBF by venous occlusion plethysmography. This device, the F2001, uses a new and sensitive technique for measuring forearm circumference, is relatively compact, and has been validated for use in assessing capillary permeability (6). The important finding of this study was a demonstration of comparable repeatability, as measured by differences in blood flow between left and right arms, when the F2001 was used compared with a standard mercury-in-Silastic device, the HEC4. In addition, there was no systemic bias between the devices as assessed by Bland-Altman analysis (3).

**Stimuli to effect changes in blood flow.** To effect small physiological increases and decreases in blood flow in the two arms, we used SCWT and LBNP, respectively. These techniques are well characterized and widely used for this purpose. However, they produced only small changes of blood flow, with generally a  $<30\%$  change. Because plethysmography techniques are often used to detect much larger changes in blood flow, especially when responses to exogenously administered vasodilators and vasoconstrictors are examined (2, 12), it was necessary to seek an additional stimulus to effect greater increases in blood flow than seen with SCWT. Here, an established ischemia model was used, and the POH response was assessed (16) to graded ischemia. This achieved our aim by producing increases of local FBF of  $\sim 800\%$  after 13 min of occlusion, equivalent to responses to potent vasodilator agents in the forearm.

**Comparison between arms.** In studies with SCWT and LBNP, we compared responses between arms, both for studies of repeatability and systematic bias. This can be justified from many studies showing that the forearm response to systemic stimuli affects both arms equally. Indeed, this is one of the principles underlying the use of the opposite arm as a control in plethysmographic studies (1, 9). Importantly, data from this study confirm the similarity in responses between arms, with only an  $\sim 1\%$  difference in percentage change in FBF from

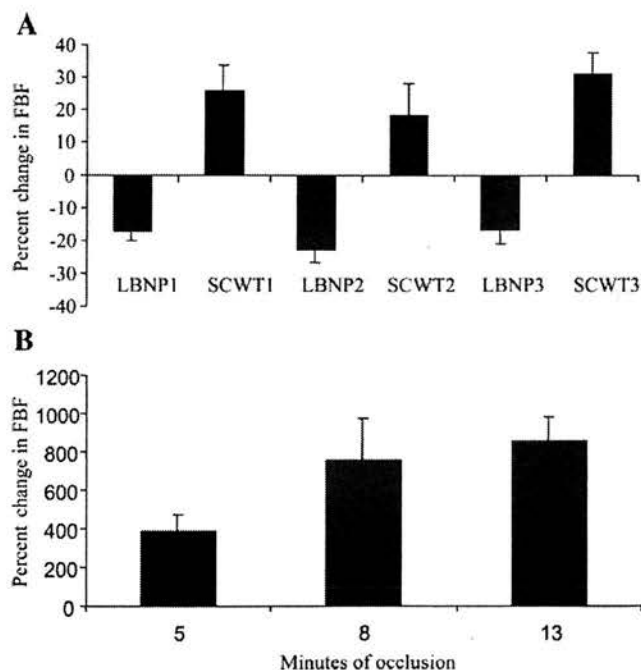


Fig. 2. A: effect of lower body negative pressure (LBNP) and Stroop's color word test (SCWT) on forearm blood flow (FBF). B: effect of postocclusive hyperemia on FBF. Changes are expressed as percentage change from the preceding baseline.

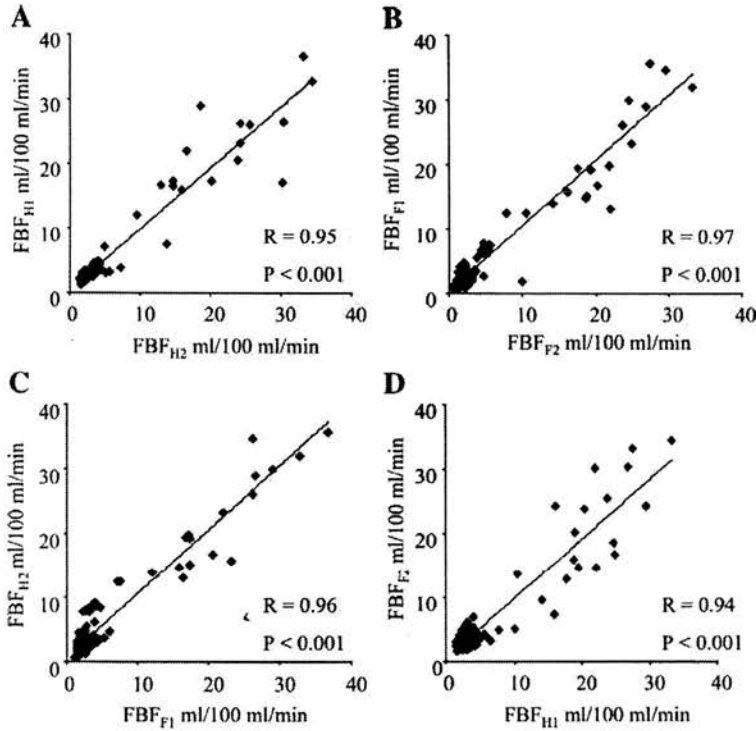


Fig. 3. Correlation graphs of Hokanson EC4 (right arm) vs. Hokanson EC4 (left arm) ( $H_1$  vs.  $H_2$ ; A), Bland-Altman comparison of Filtrass 2001 (right arm) vs. Filtrass 2001 (left arm) ( $F_1$  vs.  $F_2$ ; B), Bland-Altman comparison of Filtrass 2001 (right arm) vs. Hokanson EC4 (left arm) ( $F_1$  vs.  $H_2$ ; C), and Bland-Altman comparison of Hokanson EC4 (right arm) vs. Filtrass 2001 (left arm) ( $H_1$  vs.  $F_2$ ; D).

baseline between left and right arms to SCWT and LBNP when measured by HEC4.

In the case of POH, however, the stimulus is local and not systemic, and there may be differences between arms related

to hand dominance. Therefore, in this situation, only comparisons within one arm were made in the present study to avoid any risk of obtaining a falsely low measure of repeatability.

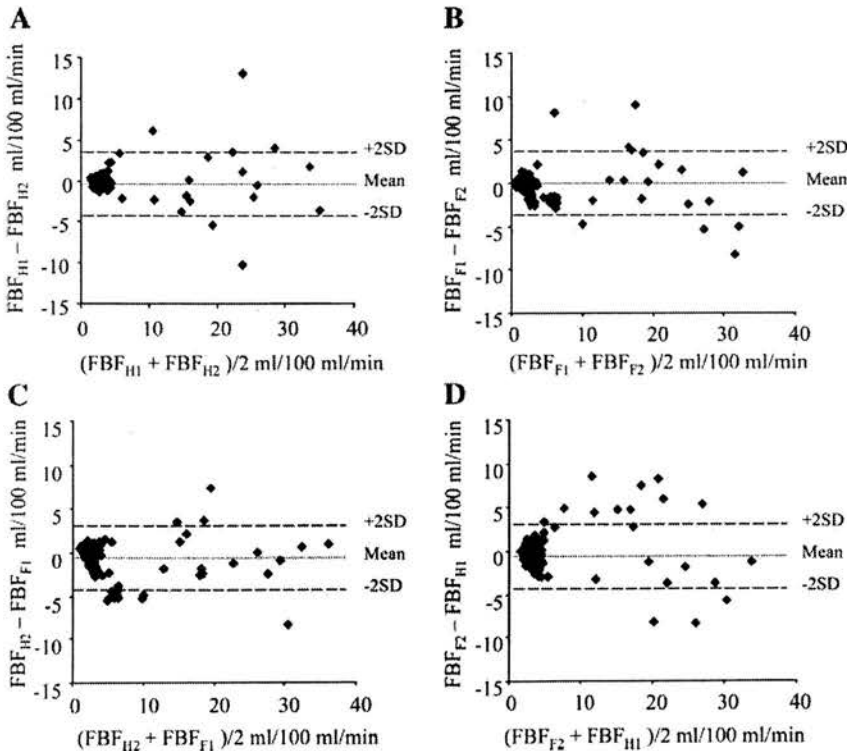


Fig. 4. Bland-Altman comparison of Hokanson EC4 (right arm) vs. Hokanson EC4 (left arm) ( $H_1$  vs.  $H_2$ ; A), Bland-Altman comparison of Filtrass 2001 (right arm) vs. Filtrass 2001 (left arm) ( $F_1$  vs.  $F_2$ ; B), Bland-Altman comparison of Filtrass 2001 (right arm) vs. Hokanson EC4 (left arm) ( $H_2$  vs.  $F_1$ ; C), and Bland-Altman comparison of Hokanson EC4 (right arm) vs. Filtrass 2001 (left arm) ( $F_2$  vs.  $H_1$ ; D). Light dotted lines indicate mean values, and dashed lines indicate 95% confidence limits.



**Comparison between devices.** The main aim of this study was to explore the comparability between devices for the measurement of blood flow by plethysmography. Because there is no readily usable gold standard for measuring FBF, we chose to compare the F2001 with one of the most widely used devices in current use, the HEC4. Many studies using forearm plethysmography involve multiple measurements of blood flow in the same individual to be performed on separate occasions. Therefore, a high degree of repeatability is required across a wide range of blood flows. LBNP and SCWT could be used to examine small decreases and increases in blood flow, respectively, and POH could be used to examine large increases. The study design allowed within-device as well as between-device comparison of effects of external stimuli on blood flow to assess both repeatability and systematic bias.

The studies show a high level of correlation (>90%) between devices that was similar to that for within-device correlations. More importantly, there was no difference in baseline blood flow or in the response to the various stimuli (LBNP, SCWT, and POH) between devices and no difference between measurements even at the high blood flows associated with POH. Furthermore, Bland-Altman analysis revealed no systematic bias. These data, taken together, show a high level of repeatability that is sufficient to allow either device to be used for plethysmographic studies.

**Potential advantages of the F2001.** The recently developed F2001 is designed to measure FBF and can also be used to measure capillary permeability. The F2001 device is more compact and portable than the HEC4. This may be of advantage in certain circumstances, such as in intensive care units or in studies where the equipment has to be moved between locations. Both types of strain gauge were easy to use, although the F2001 required greater manual dexterity to insert the monofilament into the body of the LVDT. With either device, the subject must remain still throughout the study, because movement of the forearm can dislodge the strain gauges. However, the HEC4 strain gauge may be simply replaced with little disruption to the study, whereas disruption of the F2001 strain gauge requires recalibration. This might be problematic if displacement occurred during a critical part of an interventional study. In terms of mechanical reliability, one F2001 strain gauge failed during the study (total of 40 study days), whereas there were no failures of the HEC4 strain gauges. The upper cuff air inlet of the F2001 mechanically interfered with the strain gauge on inflation, and the rate of arm and wrist cuff inflations was slower than the HEC4 (3–4 vs. <1 s). Slowly inflating cuffs can result in venous engorgement if arterial occlusion is significantly delayed, which could then cause discomfort and potentially affect results. However, neither of these problems appeared to occur in our study, suggesting the slower rate of cuff inflation is not of practical importance, even at high flows. Although there may be local variations, there was no major cost difference between the two devices. In addition, unlike the HEC4, the F2001 does not use mercury in its construction and, therefore, has the advantage that its use would not be restricted in the future were the use of mercury in medical instruments to be banned (15).

**Potential limitations of the study.** For ethical reasons, it was not possible to assess intra-arterial responses to vasodilator and constrictor agents directly in these studies. Therefore, postischemic hyperemia was used as the surrogate stimulus for

effecting large increases in flow. It is necessary, and probably reasonable, to make the assumption that these observations can be extrapolated to such studies. Similarly, studies here were performed in the forearm circulation, and we cannot be certain that they also apply to the calf, although this again would seem a reasonable assumption.

In summary, the F2001 seems a promising and reliable new addition to the systems available to measure blood flow by strain-gauge plethysmography, with potential advantages in terms of size, mobility, data analysis, and its capacity to be used to measure capillary permeability.

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# Endothelins and their inhibition in the human skin microcirculation: ET<sub>[1-31]</sub>, a new vasoconstrictor peptide

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## Aims

Endothelin-1 (ET-1<sub>[1-21]</sub>) is an extremely potent vasoconstrictor in the human skin microcirculation and is generated from larger precursor peptides. The aims of the present study were to assess the vasoactive effects of these precursors as well as endothelin blockade in the human skin microcirculation, *in vivo*.

## Methods

Six healthy volunteers received intradermal injections of a range of doses of big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub>, ET-1<sub>[1-21]</sub>, BQ-123 (ET<sub>A</sub> receptor antagonist), BQ-788 (ET<sub>B</sub> receptor antagonist), phosphoramidon [endothelin converting enzyme (ECE) inhibitor] or saline control (0.9%). Skin blood flow (SBF) was measured using standard laser Doppler flowmetry.

## Results

Big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub> and ET-1<sub>[1-21]</sub> reduced SBF when compared with saline control ( $P < 0.01$  for all). Big ET-1<sub>[1-38]</sub> and ET-1<sub>[1-31]</sub> were less potent than ET-1<sub>[1-21]</sub> as defined by skin vasoconstriction. Phosphoramidon, BQ-123 and BQ-788, given alone, all caused vasodilatation in the human skin microcirculation ( $P < 0.01$  for all).

## Conclusions

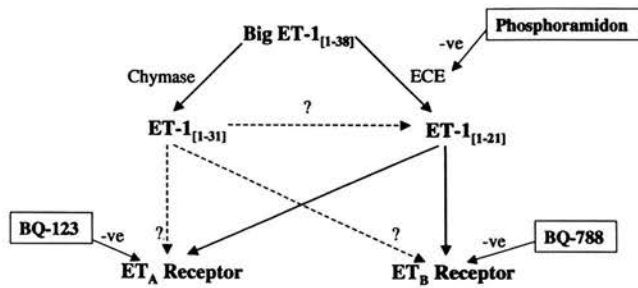
In the human skin microcirculation, big ET-1<sub>[1-38]</sub> and ET-1<sub>[1-31]</sub> are less potent vasoconstrictors than ET-1<sub>[1-21]</sub>. The effects of big ET-1<sub>[1-38]</sub> and phosphoramidon suggest the presence of endogenous ECE activity in the skin. In contrast to skeletal muscle resistance vessels, ET-1<sub>[1-21]</sub> contributes to the maintenance of skin microvascular tone through both ET<sub>A</sub> and ET<sub>B</sub> receptor-mediated vasoconstriction.

## Introduction

Endothelin-1<sub>[1-21]</sub> (ET-1) is a potent vasoconstrictor in several vascular beds including the skin microcirculation [1, 2]. It is formed by the action of endothelin converting enzyme (ECE) on its inactive precursor ET-1<sub>[1-38]</sub> (big ET-1) (Figure 1). There are two distinct endothelin receptors subtypes; ET<sub>A</sub> and ET<sub>B</sub>. Both are present on vascular smooth muscle cells mediating vasoconstriction, while ET<sub>B</sub> is also present on endothelial cells mediating vasodilatation. Thus, the cardiovascular effects of ET-1<sub>[1-21]</sub> will depend, in part, on the balance of action at these two receptors, and in particular the

balance between the vasodilating and vasoconstricting actions of the ET<sub>B</sub> receptor.

The endothelin system may be involved in the pathophysiology of several cardiovascular diseases, including hypertension [3], renal failure [4], pulmonary hypertension [5], and chronic heart failure (CHF) [6]. Blockade of the system remains an area of major interest. In particular, whether selective or dual receptor antagonism or ECE inhibition will provide the best treatment strategy remains unclear. Initial efforts have been focused mainly on endothelin receptor blockade. Endothelin receptor antagonists have benefits in patients with primary pul-



**Figure 1**

Pathway of endothelin processing. Agents in boxes are enzyme inhibitors/receptor antagonists

monary hypertension [7], although results in patients with CHF have so far been disappointing [8]. Clinical development of ECE inhibitors continues but there are, as yet, no fully published data on their clinical efficacy.

Recently, ET-1<sub>[1-31]</sub>, a new derivative of big ET-1, has been identified in humans. It is generated following the cleavage of big ET-1 at the Tyr<sub>31</sub>–Gly<sub>32</sub> bond by human chymase [9] and may represent an active intermediary in an alternative pathway of ET-1 production (Figure 1). In cultured human coronary artery smooth muscle cells [10, 11] and human mesangial cells [12], ET-1<sub>[1-31]</sub> increases intracellular calcium. ET-1<sub>[1-31]</sub> causes vasoconstriction in isolated porcine coronary arteries [13], monkey trachea [14], human umbilical arteries [15] as well as human coronary and mammary arteries [16]. Data suggest that ET-1<sub>[1-31]</sub> may be cleaved to ET-1<sub>[1-21]</sub> for its biological activity in cultured bronchial smooth muscle cells [17] and in both guinea pig [18] and human arteries [16]. However, to date there have been no *in vivo* clinical studies to assess the cardiovascular effects of ET-1<sub>[1-31]</sub>.

The aims of this study (Fig. 1) were to investigate the *in vivo* vascular effects of ET-1<sub>[1-21]</sub>, its precursors big ET-1<sub>[1-38]</sub> and ET-1<sub>[1-31]</sub>, and blockade of endogenous ET-1 activity by BQ-123 (a selective ET<sub>A</sub> receptor antagonist) [19], BQ-788 (a selective ET<sub>B</sub> receptor antagonist) [20] and inhibition of ET-1 generation by phosphoramidon (an ECE inhibitor) in the human skin microcirculation.

## Methods

### Subjects

Six healthy men (age range 20–30 years), with no risk factors for vascular disease, participated in each study. Written informed consent was obtained and studies were performed with the approval of the local research ethics committee and in accordance with the Declaration of

Helsinki. No subject was taking regular medication and all avoided medication for 1 week before each study. All subjects abstained from alcohol for 24 h and from food, caffeine and tobacco for at least 12 h before each study.

### Skin blood flow measurement

Skin blood flow was assessed using standard laser Doppler skin flowmetry (2 channel, MBF 3D; Moor Instruments Ltd, Axminster, UK) at baseline and every 2 min for the first 10 min and then every 5 min up to 60 min. Voltage output from the Doppler flowmeter was calibrated with standard flux solution (Moor Instruments Ltd) and transferred to a Macintosh personal computer (Classic II; Apple Computer Inc., Cupertino, CA, USA) with a MacLab analogue-to-digital converter and 'CHART' software (v.3.28; AD Instruments, Castle Hill, Australia). Signals were averaged over 20 s at each time point.

### Study drugs

ET-1<sub>[1-31]</sub> (Peptide Institute, Osaka, Japan), and big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-21]</sub>, BQ-123, BQ-788 and phosphoramidon (Clinalfa, Laufelfingen, Switzerland) were dissolved in physiological saline (0.9%; Baxter Healthcare Ltd, Thetford, UK), which was also used as the vehicle control. Phosphoramidon was poorly soluble, allowing a limited dose range to be examined.

### Study protocol

Subjects rested recumbent in a quiet room maintained at a constant temperature of 22–24 °C for 15 min to allow stabilization of skin blood flow. Four sites for injection were identified and marked on the volar aspect of each forearm. Care was taken to avoid underlying veins (demonstrated by high baseline Doppler signals) and arteries (demonstrated by pulsatile Doppler signals). A laser probe holder was attached to the skin using adhesive tape to reduce probe movement during the study. All study drugs were administered by 10 µl intradermal injection [0.33-mm (29.5 SWG) needle; Becton Dickinson, Dublin, Ireland]. Following dose-ranging pilot studies, subjects received, in random order, either saline control or study drug over a range of concentrations; big ET-1<sub>[1-38]</sub> (0.1–30 pmol), ET-1<sub>[1-31]</sub> (1 pmol to 0.3 nmol), ET-1<sub>[1-21]</sub> (1 amol to 1 pmol), BQ-123 (0.1–30 nmol), BQ-788 (0.1–30 nmol) and phosphoramidon (0.1–10 nmol). The maximum dose of phosphoramidon was limited by solubility.

### Data handling and statistical analysis

Results are expressed in arbitrary perfusion units (PU). Intradermal injection of saline placebo causes an increase in laser Doppler signal [1] and therefore all



results are presented as placebo corrected mean  $\pm$  SEM. Area under the curve (AUC) for the response between 0 and 30 min was used to determine potency. Potency was estimated as the dose required to cause a significant vasoconstriction in the skin compared with saline placebo. Statistical difference was tested by ANOVA with repeated measures over time and paired Student's *t*-test with correction for repeated measures (Excel 5.0; Microsoft Ltd, Seattle, WA, USA). A *P*-value  $<0.05$  was considered to be statistically significant.

## Results

Intradermal drug delivery was well tolerated by all volunteers. Transient discomfort occurred at some injection sites but was unrelated to injectate and did not persist for longer than 10 s. This discomfort was not associated with impaired tissue viability and did not appear to affect responses.

### Effect of endothelin agonists

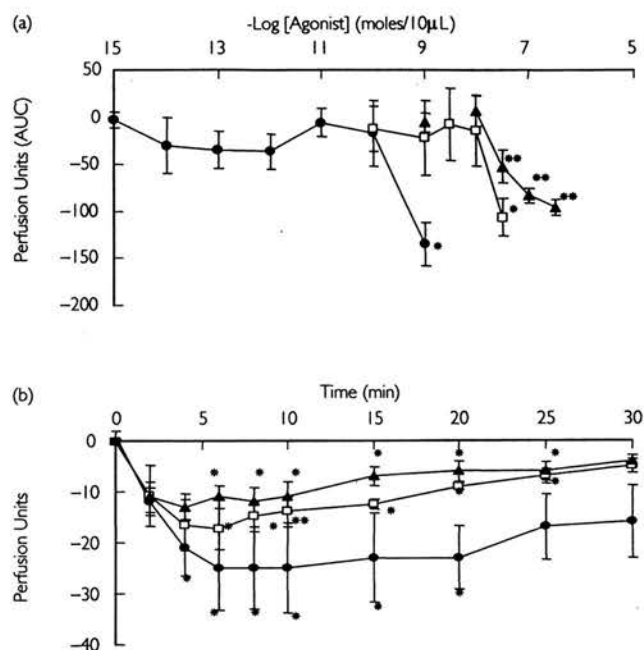
Big ET-1<sub>[1-38]</sub>, ET-1<sub>[1-31]</sub> and ET-1<sub>[1-21]</sub> caused vasoconstriction that was visually evident on the skin, causing an area of marked pallor. Big ET-1<sub>[1-38]</sub> and ET-1<sub>[1-31]</sub> were ~30-fold less potent than ET-1<sub>[1-21]</sub> (Figure 2a). Compared with control, sustained reduction in blood flow was caused by big ET-1<sub>[1-38]</sub> (30 pmol; maximum decrease  $25 \pm 8$  PU, *P* = 0.04), ET-1<sub>[1-31]</sub> (0.3 nmol; maximum decrease  $13 \pm 3$  PU, *P* = 0.04) and ET-1<sub>[1-21]</sub> (1 pmol; maximum decrease  $17 \pm 4$  PU, *P* = 0.003) (Figure 2b). At these concentrations, vasoconstriction was sustained and was still visibly present at 24 h, although the duration of response beyond 60 min was not formally assessed.

### Effect of endothelin blockade

BQ-123 and BQ-788 caused vasodilatation (Figure 3a). Compared with control, a sustained increase in blood flow was caused by BQ-123 (300 nmol; maximum increase  $30 \pm 5$  PU, *P* = 0.002) and BQ-788 (300 nmol; maximum increase  $18 \pm 5$  PU, *P* = 0.004) (Figure 3b). Compared with control, phosphoramidon caused a small increase in blood flow at the highest dose (10 pmol; maximum increase  $11 \pm 2$  PU, *P* = 0.009; Figure 3a,b).

## Discussion

In the human skin microcirculation, we have confirmed that ET-1<sub>[1-21]</sub> is a potent vasoconstrictor and shown for the first time that ET-1<sub>[1-31]</sub> and big ET-1<sub>[1-38]</sub> also cause skin vasoconstriction *in vivo*. Our results suggest that there is ECE activity in the skin as demonstrated by the vasoconstriction following intradermal administration



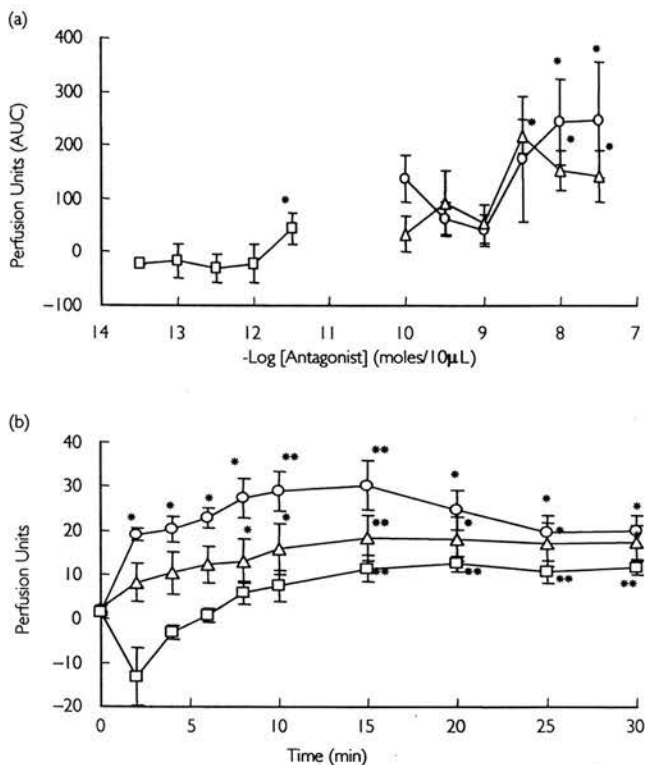
**Figure 2**

(a) Dose-response (AUC) to big ET-1<sub>[1-38]</sub> (0.1–30 pmol) (□), ET-1<sub>[1-31]</sub> (1 pmol to 0.3 nmol) (▲), and ET-1<sub>[1-21]</sub> (1 amol to 1 pmol) (●). (b) Effect of maximum dose of endothelin agonist on skin blood flow; ET-1<sub>[1-38]</sub> (30 pmol), ET-1<sub>[1-31]</sub> (0.3 nmol) and ET-1<sub>[1-21]</sub> (1 pmol).

\**P* < 0.05; \*\**P* < 0.01 vs. placebo

of big ET-1<sub>[1-38]</sub> and vasodilatation with ECE inhibition. In addition, we have confirmed that selective blockade of the ET<sub>A</sub> receptor caused skin microvascular vasodilatation [2] and shown directly that selective blockade of the ET<sub>B</sub> receptor also results in vasodilatation. In contrast to observations in resistance vessels, this suggests that ET<sub>B</sub> receptors in the skin contribute to ET-1-mediated vasoconstriction not only in arterial disease [2] but also in healthy blood vessels.

The novel finding that ET-1<sub>[1-31]</sub> is a vasoconstrictor is of interest, and the first evidence of its vasoactive properties *in vivo* in man. If ET-1<sub>[1-31]</sub> is converted to ET-1<sub>[1-21]</sub> by a non-ECE pathway and if this contributes importantly to ET-1 generation, then specific receptor blockade may offer greater functional inhibitory activity than ECE inhibition. Alternatively, ET-1<sub>[1-31]</sub> may have vasoconstricting activity of its own at endothelin receptors. Recently, the vasoconstricting effects of ET-1<sub>[1-31]</sub> have been shown to be mediated via the ET<sub>A</sub> receptor in rabbit renal resistance vessels [21], an effect which was unaffected by phosphoramidon. Although ET-1<sub>[1-31]</sub> is less potent than ET-1<sub>[1-21]</sub> as a vasoconstrictor, local production of ET-1<sub>[1-31]</sub> may occur specifically in tissues that express human chymase, such as from the mast cells



**Figure 3**

(a) Dose-response (AUC) to BQ-123 (0.1–30 nmol) (○), BQ-788 (0.1–30 nmol) (△), phosphoramidon (0.1–10 nmol) (□). (b) Effect of maximum dose of endothelin blockade on skin blood flow; BQ-123 (30 nmol), BQ-788 (30 nmol), phosphoramidon (10 nmol). \* $P < 0.05$ ; \*\* $P < 0.01$  vs. placebo

within the shoulder region of coronary atheromatous plaques [22, 23]. Therefore, generation of ET-1<sub>[1–31]</sub> could contribute to coronary artery spasm at the time of plaque rupture. Indeed, ET-1<sub>[1–31]</sub> may be cleaved to ET-1<sub>[1–21]</sub> for its biological activity in cultured bronchial smooth muscle cells [17] and in both guinea pig [18] and human arteries [16].

The fact that big ET-1<sub>[1–38]</sub> has affinity for the endothelin A receptor which is 1000-fold less than ET-1<sub>[1–21]</sub> [20], and that big ET-1<sub>[1–38]</sub> was ~30-fold less potent than ET-1<sub>[1–21]</sub>, makes it unlikely that big ET-1<sub>[1–38]</sub> caused a major direct vasoconstrictor action and suggests that there is some conversion of big ET-1 to ET-1<sub>[1–21]</sub> in the skin. It has previously been demonstrated that big ET-1<sub>[1–38]</sub> is vasoactive in human arteries [3] but not veins [25], and that this vasoconstriction can be blocked by ECE inhibition [26]. The presence of ECE activity in the skin is further supported in this present study by the vasodilatory effects of phosphoramidon. This is also consistent with preliminary clinical studies

where systemic ECE inhibition caused systemic vasodilatation and a reduction in blood pressure [27]. In our study there appeared to be a transient vasoconstriction to phosphoramidon, although this was not consistent and did not reach statistical significance. This observation may warrant further investigation.

Endothelin antagonists have been studied in the skin by other groups. The selective ET<sub>A</sub> receptor antagonist PD147953 and the nonselective endothelin receptor antagonist PD145065 caused vasodilatation and attenuated the vasoconstrictor effects of ET-1<sub>[1–21]</sub> in the human skin *in vivo* [1, 28]. BQ-123 is a selective ET<sub>A</sub> receptor antagonist and has been shown in many vascular beds [29] to be a vasodilator by blocking the effects of ET-1<sub>[1–21]</sub>. There are few data on the effects of endothelin antagonists in the skin [1, 2, 28]. The present study demonstrates that BQ-123 causes vasodilatation, confirming that ET-1 contributes to the maintenance of basal vascular tone in the skin of healthy volunteers. BQ-788 also caused skin vasodilatation. This result was unexpected, as selective ET<sub>B</sub> receptor antagonism causes vasoconstriction in skeletal muscle [30] and when given systemically [31]. In addition, previous data on the skin microcirculation have demonstrated that ET-1 mediates vasoconstriction via the ET<sub>A</sub> receptor with little contribution from the ET<sub>B</sub> receptor [28, 32]. However, our data indicate that the dominant effect of ET-1<sub>[1–21]</sub> on the ET<sub>B</sub> receptor is vasoconstriction in the human skin microcirculation, and that blockade of the ET<sub>B</sub> receptor results in vasodilatation. It is possible that some of this effect may be a result of displacement of ET-1 from ET<sub>B</sub> receptors and a reduction of available ET<sub>B</sub> receptor binding sites, thereby increasing vasoconstriction via the ET<sub>A</sub> receptor. This finding may require further investigation.

Endothelin receptor antagonists have proven benefits in patients with primary pulmonary hypertension [7] and reduce blood pressure in hypertensive patients [33]. To date, clinical trials investigating the potential benefits of endothelin receptor antagonists in CHF have been disappointing [8]. ECE inhibitors inhibit endothelin production and provide a potential alternative to endothelin receptor antagonism. Unlike receptor blockade, which can increase plasma ET-1<sub>[1–21]</sub> concentrations, ECE inhibitors have the advantage that they block ET-1<sub>[1–21]</sub> production, thus reducing plasma ET-1<sub>[1–21]</sub> concentrations and leaving clearance receptors unblocked. However, the effects of raised plasma concentration of ET-1<sub>[1–21]</sub>, associated with ET<sub>B</sub> receptor blockade [30], may not be relevant if ET<sub>A</sub> receptors are also blocked. However, if there are clinically significant non-ECE pathways of production with vasoactive intermediary

peptides such as ET-1<sub>[1-31]</sub>, then ECE inhibition may be a less attractive treatment strategy.

While the doses of peptides used in these experiments are known, the concentration when injected into the skin cannot be measured, due to the unknown volume of distribution in the skin. However, as the same volume of injectate was used for each dose, comparing the relative potencies of each peptide is probably justified. Furthermore, true potency may vary depending on whether compounds are full or partial agonists. Highly selective antagonists of each receptor were not available to us. Although BQ-788 might have blocked ET<sub>A</sub> receptor function, its 100-fold greater affinity for the ET<sub>B</sub> receptor [34] compared with the ET<sub>A</sub> receptor, and the equal potency of BQ-123 and BQ-788 in this study, suggest that BQ-788 does indeed mediate vasodilatory effects via blockade of the ET<sub>B</sub> receptor. Another limitation is that phosphoramidon is not only an inhibitor of ECE but also an inhibitor of neutral endopeptidase (NEP). However, whether in health or cardiovascular disease [3, 35, 36], the inhibition of NEP tends to cause vasoconstriction and therefore if anything lead to an underestimate of the vasodilator effects of selective ECE inhibition.

In summary, the discovery of a vasoactive intermediary endothelin peptide may have importance for the further development of endothelin blockers as clinical therapies. The skin microcirculation provides an opportunity to investigate the vasoactive properties of a compound, *in vivo*, in a relatively safe manner due to the small doses which are administered. However, further studies are required to determine whether ET-1<sub>[1-31]</sub> is vasoactive in other vascular beds. In particular it will be of interest to note whether ET-1<sub>[1-31]</sub> causes vasoconstriction in resistance blood vessels.

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## Endothelin-1 Antagonises Beta-Adrenergic Stimulation in Human Right Atrial Myocardium

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Plasma concentrations of endothelin-1 (ET-1) are elevated in chronic heart failure (CHF) and correlate with a poor prognosis [1]. Short-term systemic ET blockade in CHF patients causes potentially beneficial haemodynamic effects; increasing cardiac output and reducing vascular resistance [2]. However, the initial results from longer term clinical trials have been disappointing. In the ENABLE and EARTH clinical trials preliminary reports indicate that there was no major benefit when an endothelin receptor antagonist (ETRA) was added to conventional heart failure therapy.

Beta-blockers improve mortality in CHF patients and while the mechanisms for these benefits are not fully understood they may involve beta<sub>1</sub>-adrenoreceptor upregulation. Beta<sub>1</sub>-adrenergic stimulation increases adenylate cyclase (AC), increasing cAMP, activating protein kinase A, resulting in phosphorylation of several intracellular proteins ultimately leading to an increase in intracellular calcium. The positive inotropic effect of ET-1 is mediated predominantly via the ET<sub>A</sub> receptor resulting in activation of protein kinase C via G proteins. However, in isolated myocardial membrane studies ET-1 also inhibits AC and may therefore inhibit beta-adrenergic stimulation [3, 4]. This potentially important antagonism between the two systems has not been previously studied at a functional level.

### Methods

12 right atrial appendage biopsies were harvested at the time of coronary artery bypass surgery and placed in cold cardioplegic solution ([mM]; NaCl 130, KCl 5.4, NaHPO<sub>4</sub> 0.56, MgCl<sub>6</sub>H<sub>2</sub>O 3.5, CaCl 2, Glucose 10, HEPES 5, 2,3-butanedione-monoxime 30 [mM] corrected to pH 7.4 with NaOH). Free running trabeculae were mounted for electrical stimulation at 3 Hz at a voltage  $\approx$  10 % above threshold and allowed to stabilise for 1 hour in a vertical 7 ml chamber (World Precision Instruments, UK) containing modified Tyrodes (NaCl 130, KCl 5.4, NaHPO<sub>4</sub> 0.56, MgCl<sub>6</sub>H<sub>2</sub>O 3.5, CaCl 2, Glucose 10, HEPES 5 [mM] corrected to pH 7.4 with NaOH and continuously bubbled with 100 % O<sub>2</sub> at 35 °C. ET-1 (Neosystem SNPE England, UK) was reconstituted in 0.9 % saline and isoprenaline (Sigma-Aldrich Chemicals, UK), a beta<sub>1</sub>-adrenoreceptor agonist, was dissolved in physiological solution with ascorbic acid (1 mM) to reduce oxidation. Drug concentrations were chosen from previous dose ranging studies. Trabeculae were exposed to either isoprenaline (10 nM), ET-1 (10 nM) or isoprenaline followed by ET-1. When possible two trabeculae were dissected from a single atrial appendage.

Statistical difference was tested by Student's t-test (Excel 5.0, Microsoft). A value of  $p < 0.05$  was considered to be statistically significant. All values are expressed as mean  $\pm$  SEM.

The study was approved by the local research ethics committee and written informed consent obtained from each subject.

### Results

ET-1 (10 nM) increased force by  $12.6 \pm 5.0$  %,  $n = 5$ ,  $p = 0.04$ . This was preceded by a transient non-significant decrease in force ( $-3.6 \pm 1.9$  %,  $p = \text{ns}$ ). Isoprenaline (10 nM) increased force from baseline  $65.7 \pm 29.2$  %,  $n = 5$ ,  $p = 0.01$ . The increase in force with isoprenaline was of more rapid onset, with a maximal effect seen after 5 min (Fig. 1). When added to maximally beta-adrenergic stimulated trabeculae, ET-1 significantly attenuated the effect of isoprenaline causing a reduction in force ( $96 \pm 39$  %,  $n = 5$ ,  $p < 0.01$ ) (Fig. 2).

### Discussion

Both ET-1 and isoprenaline increased isometric force in human RA myocardium. ET-1 increased force following a transient negative inotropic response, and although this has been described by other groups [4, 5] the mechanism remains unclear. Isoprenaline increased force, which was of faster onset and larger magnitude than ET-1. We have observed, for the first time, that ET-1 functionally attenuates the inotropic effects of isoprenaline in human myocardium. This reduction in force was observed after maximal stimulation of the beta-adrenergic system. The underlying mechanism is likely to be interaction at the level of AC as seen in isolated membrane studies where ET-1 was shown

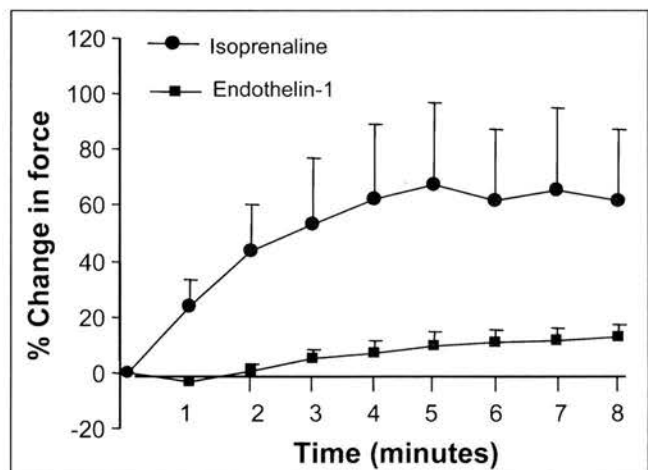


Figure 1. Effect of isoprenaline (10 nM) and endothelin-1 (10 nM) on force of contraction of human right atrial trabeculae

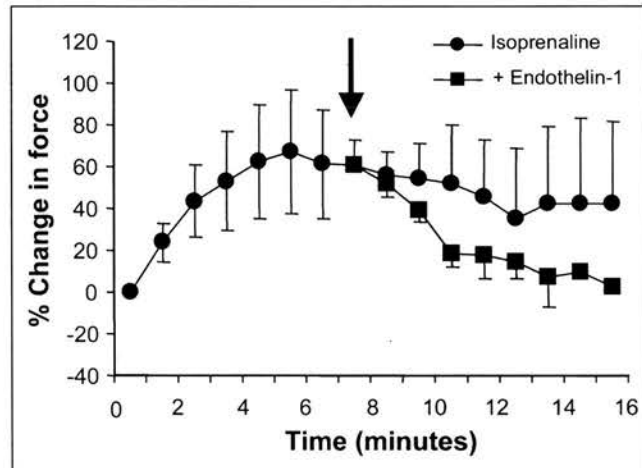
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to inhibit the effect of  $\beta_1$ -adrenoreceptor activation on this enzyme.

This study has some limitations in that we were unable to study normal human ventricular myocardium due to a lack of suitable samples. Previous isolated membrane studies have confirmed differences between atrial and ventricular tissue so ET-1 may not inhibit AC in ventricular tissue [5]. In addition, the concentrations of ET-1 used were higher than those in the plasma of CHF patients but may be similar to those occurring at a local level in myocardium.



**Figure 2.** Effect of endothelin-1 (10 nM) on isoprenaline (10 nM) stimulated human right atrium; black arrow indicates the addition of endothelin-1 (10 nM)

The interaction between endothelin and adrenergic systems in the human myocardium may be significant in patients with CHF where both systems are activated. ET-1 may protect from catecholamines over-stimulation and may be anti-arrhythmic. With the possible introduction of ETARs for the treatment of CHF the physiological and patho-physiological interactions between these two systems may be of clinical importance as unintended deleterious effects may occur with ET blockade in the absence of  $\beta_1$ -adrenoreceptor blockade.

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The authors have no conflicts of interest.

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## Validation of laser Doppler flowmetry coupled with intra-dermal injection for investigating effects of vasoactive agents on the skin microcirculation in man

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**Abstract Objective:** To determine the reproducibility of laser Doppler flowmetry coupled with intra-dermal saline delivery.

**Methods:** Delivery of saline was judged visually by two operators ( $n=100$ ), using a graduated syringe (Becton-Dickinson), by expelling saline onto a weighing boat. Volume was assessed by weight. Skin blood flow following intra-dermal injection of saline was assessed in 18 healthy volunteers; 10 attended twice to assess between-day reproducibility, and 8 attended once to assess between-site reproducibility. Results are expressed as mean value  $\pm$  SEM and 95% confidence interval for mean differences.

**Results:** There was no difference between operators in mean injection weight, both weights being  $10.3 \pm 0.1$  mg (mean difference 0.08, 95% confidence interval, CI  $-0.23$  to  $0.39$  mg;  $n=100$ ,  $P=0.9$ ). Intra-dermal saline caused a nine-fold increase in blood flow ( $0.03 \pm 0.003$  to  $0.27 \pm 0.02$  perfusion units, PU;  $n=18$ ,  $P<0.001$ ). This response had a rapid onset, with the maximal effect seen at 4 min and a duration of greater than 30 min. There was no difference in the magnitude of the response between the dominant and non-dominant arms, AUC was  $2.9 \pm 0.4$  and  $2.9 \pm 0.4$ , respectively (mean difference  $-0.05$ , 95% CI  $-0.8$  to  $0.73$  PU;  $n=18$ ,  $P=0.93$ ). However, there was a trend towards differences between study visits 1 and 2: AUC was  $3.2 \pm 0.6$  and  $2.0 \pm 0.5$ , respectively (mean difference 1.2, 95% CI  $-0.03$  to  $2.43$  PU;  $n=10$ ,  $P=0.7$ ). There was no difference in the

magnitude of responses between different sites on the forearm ( $n=64$ ,  $P=0.6$ ).

**Conclusions:** These studies demonstrate that the technique of laser Doppler flowmetry coupled with intra-dermal injection is a safe, well-tolerated technique with good reproducibility. A trend towards reduced between-day reproducibility emphasizes the importance of vehicle control sites when investigating the effects of vasoactive compounds. This technique provides a reliable method for the intra-dermal delivery of drugs, despite the direct effect of injection of saline on blood flow.

**Keywords** Skin blood flow · Laser Doppler flowmetry · Intra-dermal injection

### Introduction

Laser Doppler flowmetry is a well-validated technique used for the investigation of the effects of vasoactive substances on the skin microcirculation [1, 2, 3, 4, 5, 6, 7, 8, 9, 10]. It has several potential advantages over other techniques for the initial study of drugs in humans in that it is minimally invasive and relatively safe, because it uses very small doses of study compounds with a mainly local action. It allows separate sites on the skin to be studied simultaneously and thus the investigation of a range of concentrations on the same occasion. While it is possible to deliver some vasoactive substances such as acetylcholine and noradrenaline by skin iontophoresis, many peptides cannot be delivered by this means due to their large size, poor solubility, or lack of electrical charge. In these situations, intra-dermal injection can be used. Physiological saline is commonly used in studies as a drug vehicle, and there are a variety of high-precision syringes that can be used for intra-dermal injection. However, these are expensive and when used in human studies can, by necessity, be used only for a single subject. In the current studies, we used standard clinical insulin syringes with a 29.5 SWG-gauge needle

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to administer local intra-dermal injections. The advantages of these syringes are that they are relatively inexpensive and easy to use. These syringes have been used by other groups in skin blood flow (SBF) studies [7, 8, 9] but have not been validated for use for intra-dermal injection in pharmacological studies. There are potential sources of error in the injection technique, because the plunger is depressed by only 1 mm to deliver 10  $\mu$ l and is judged visually. The reproducibility of intra-dermal delivery in terms of volume delivered and the effect of intra-dermal saline injection on SBF using these syringes has not been reported previously.

The aim of these studies was to determine the intra- and inter-operator reproducibility of saline administration in terms of injection volume and to determine reproducibility of SBF responses to intra-dermal injection of saline.

## Methods

### Subjects

Eighteen right-handed, healthy men (22–45 years) were studied. Studies were performed with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki of the World Medical Association. Written informed consent was obtained from each subject before entry to the study. None of the subjects were taking regular medication and all avoided any medication for 1 week prior to study. All subjects abstained from alcohol for 24 h and from food, tobacco, and caffeine-containing drinks for at least 12 h before each study.

### Injection volume

Graduated 29.5 SWG syringes (Becton-Dickinson, Dublin, Ireland) were used for saline delivery. Each 1-mm graduation on the 0.5-ml syringe represents 10  $\mu$ l, thus, to deliver this volume, the syringe plunger was depressed by 1 mm. The reproducibility of volume delivered was assessed by injecting 10  $\mu$ l saline, judged by depressing the syringe plunger by one graduation, onto a weighing boat placed on a balance (Mettler Toledo MT5). This balance has an accuracy and precision of 1  $\mu$ g and therefore can measure changes of 1 nl assuming a specific gravity of saline of 1.00. A new syringe was used for each saline delivery and care was taken, as in our clinical studies, to expel any air bubbles from the syringe before injecting. Two operators each performed blind 100 injections.

### Laser Doppler flowmetry and intra-dermal injection

Subjects lay supine with the arms supported and the volar aspect of the forearm facing upwards. Sites for injection were chosen, taking care to avoid underlying arteries (assessed by palpation and pulsatile Doppler signal) or veins (assessed visually and by high-baseline Doppler signal). Four laser probe holders were attached to the skin of each forearm with adhesive tape to ensure no displacement of the probe during the study.

### Protocol

Studies were performed in a quiet, temperature-controlled, draught-free room (23–25°C). Each subject was allowed to rest for at least 20 min before the study protocol was started. Baseline recordings were made. Subjects then received a number of intra-dermal injections of 10  $\mu$ l saline (0.9%; Baxter Healthcare, Thetford, UK) and laser Doppler signal recorded every 2 min until

10 min and every 5 min until 30 min. To assess between-day reproducibility, ten subjects attended for two study visits. To assess between-site reproducibility, eight of the subjects received four intra-dermal injections of saline on the volar aspect of each forearm.

### Data handling and statistical analysis

Increases in weight on the balance following saline injection were recorded manually and entered onto a spreadsheet (Excel v5.0; Microsoft). The accuracy was assessed by mean values and reproducibility by assessing the spread of results. For the SBF studies, analogue voltage output from the laser Doppler flowmeter was processed by a MacLab analogue-to-digital converter and Chart v3.3 software (AD Instruments, Castle Hill, Australia), and further analysis was performed off-line. The average signal for 30 s at each time point was recorded and entered into a spreadsheet (Excel v5.0; Microsoft). Area under the curve (AUC) over 30 min was calculated for each SBF response curve and used to determine differences between them and expressed in arbitrary perfusion units (PU). These were assessed using the method of Bland and Altman [11]. Bland-Altman analysis allows the assessment for agreement and systematic bias. Coefficients of reproducibility were determined for 95% confidence intervals (CI). Results are expressed as mean  $\pm$  SEM and 95% CI for mean differences. Statistical analysis was performed using Student's *t*-test and single-factor ANOVA for between-site reproducibility. Statistical significance was taken at the 5% level.

## Results

### Accuracy and reproducibility of injection volume

Two operators performed 100 injections each. There was no difference in mean injection weight between operators, both weights being  $10.3 \pm 0.1$  mg (mean difference 0.08, 95% CI  $-0.23$  to  $0.39$  mg;  $n = 100$ ,  $P = 0.9$ ).

### Tolerability of intra-dermal drug delivery

The technique was well tolerated by subjects, with only mild discomfort experienced during the injection of saline at some of the sites. This discomfort was variable in intensity. In the majority of subjects, the trauma from intra-dermal injection did not leave any discernable mark on the skin by the end of the study, although several injections did cause a small degree of bleeding along the track of the needle. This did not appear to affect the results in terms of reproducibility.

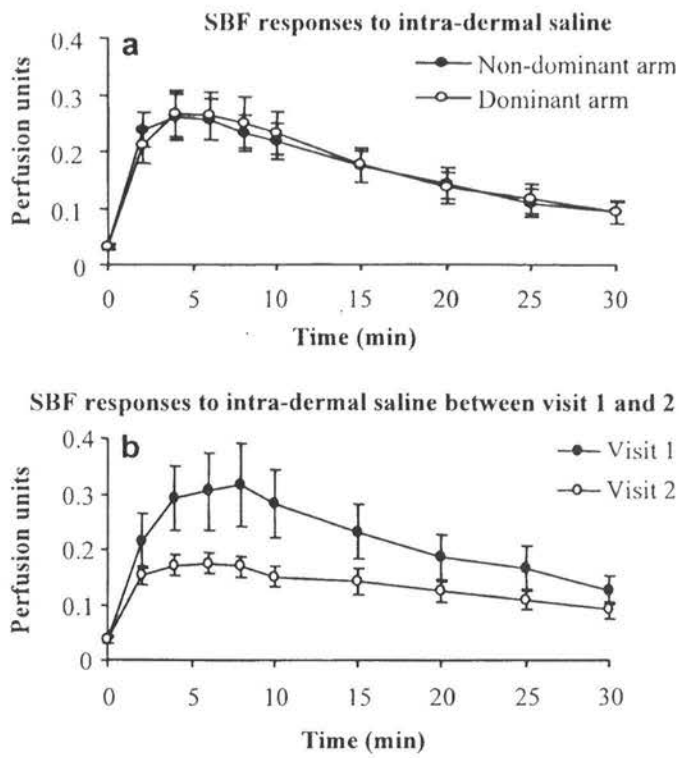
### Skin blood flow responses: effect of intra-dermal injection of saline

Saline caused a nine-fold increase in SBF ( $0.03 \pm 0.003$  to  $0.27 \pm 0.02$  PU;  $n = 18$ ,  $P < 0.001$ ). This effect was rapid in onset, with maximal response seen at 4 min (Fig. 1a).

### Within-subject same-day reproducibility: between-site reproducibility

The area under the curve was constructed for the responses at four different sites on each forearm in eight





**Fig. 1** **a** Effect of intra-dermal saline on skin blood flow (SBF) between dominant and non-dominant arm on the same study visit. **b** Effect of intra-dermal saline on SBF on the dominant arm on the different study visits

subjects. There was no difference in the magnitude of responses between sites on the forearm as assessed by AUC ( $n = 64$ ,  $P = 0.6$ ; Fig. 2).

#### Between-arm reproducibility

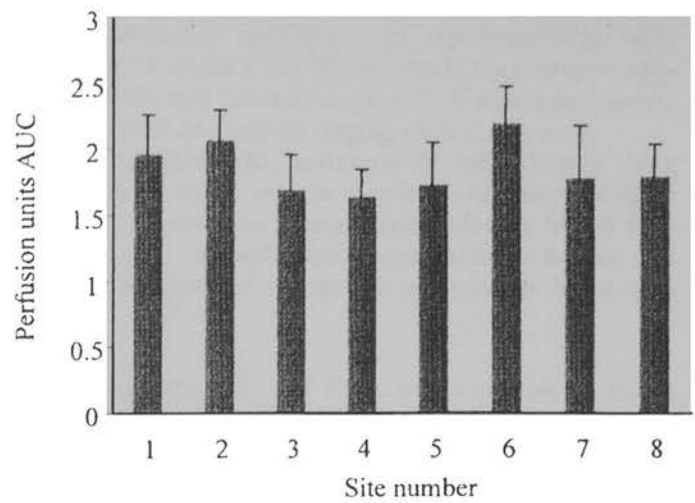
There was no difference in the magnitude of the response between the dominant and non-dominant arms, AUC was  $2.9 \pm 0.4$  and  $2.9 \pm 0.4$ , respectively (mean difference  $-0.05$ , 95% CI  $-0.8$  to  $0.73$  PU;  $n = 18$ ,  $P = 0.93$ ; Fig. 1a). Bland-Altman analysis was performed demonstrating no systematic bias and a co-efficient of reproducibility of 3.54 (Fig. 3a).

#### Between-day reproducibility

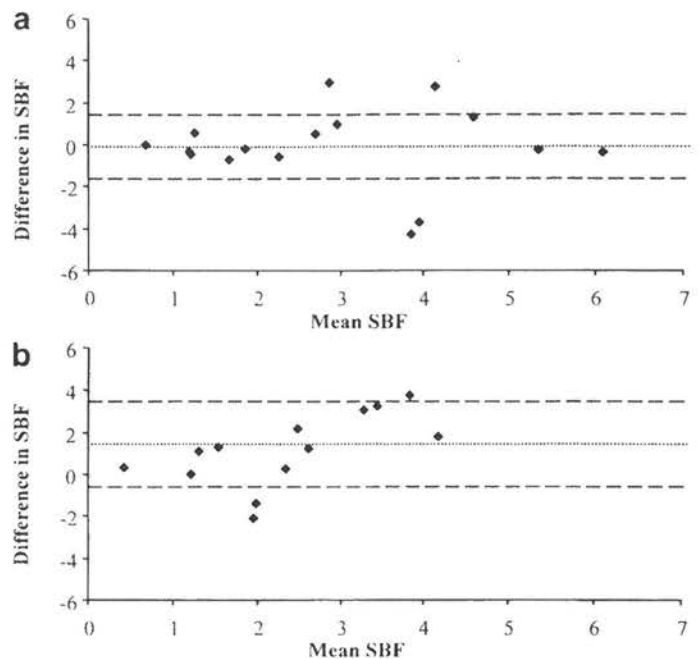
There was a trend towards a difference between study visits 1 and 2: AUC was  $3.2 \pm 0.6$  and  $2.0 \pm 0.5$ , respectively (mean difference 1.2, 95% CI  $-0.03$  to  $2.43$  PU;  $n = 10$ ,  $P = 0.7$ ; Fig. 1b). Bland-Altman analysis was performed and tended to show systematic bias and a co-efficient of reproducibility of 3.48 (Fig. 3b).

#### Discussion

In this study we have demonstrated that saline delivery using the Becton-Dickinson syringe is accurate and



**Fig. 2** Simultaneous effect of intra-dermal saline injection on SBF between 8 sites on the same day. Sites 1–4 were chosen on the dominant arm (numbered proximal to distal on forearm), sites 5–8 were chosen on the non-dominant arm (numbered proximal to distal on forearm) AUC Area under curve



**Fig. 3a, b** Bland Altman plots for **a** between-arm reproducibility and **b** between-day reproducibility

repeatable with low intra-operator variability. In addition, we have demonstrated that intra-dermal injection of saline causes a clear increase in Doppler signal but that the magnitude of this increase is similar between different sites on the forearm. There is good within-day and between-arm reproducibility.

There was occasionally mild discomfort, of variable intensity, experienced by the subjects at the time of injection. However, there did not appear to be any pattern to explain the fact that some sites developed more pain than others. Although the non-uniform

distribution of cutaneous nerves may explain this finding, this was not formally assessed but did not appear to affect the results in terms of reproducibility.

While there is often greater interest in the systemic effects of vasoactive compounds, there are potential risks with administration of systemic doses of vasoactive compounds. The use of local techniques such as intra-dermal administration with laser Doppler microcirculatory blood flow measurement has allowed the relatively safe observation of the *in vivo* effects of vasoactive compounds, without causing confounding compensatory systemic effects. While the SBF may be under different mechanisms of control to other vascular beds and responses can differ [12], in general, effects of compounds in the skin have been reflected in other less-accessible vascular beds [7, 8]. Therefore, the skin microcirculation offers a safe, well-tolerated, easy-to-use approach to the initial investigation of vasoactive compounds.

The Becton-Dickinson syringe is commonly used to deliver intra-dermal injections in clinical practice. Although it has the advantage that it is inexpensive and easy to use, its accuracy has not previously been described. Here, we found good agreement between operators as seen by mean values that were similar and close to the intended volume of 10  $\mu$ l, with most injections very close to this volume. We conclude that this syringe can be used to deliver intra-dermal injections in a clinical research setting with sufficient accuracy and reproducibility.

Intra-dermal injection of saline causes an increase in laser Doppler signal. In this study, we have demonstrated that the technique of laser Doppler flowmetry coupled with intra-dermal injection is a repeatable technique and that responses between subjects are similar. There was no difference in SBF in response to intra-dermal saline injection between sites on the same arm or in the same patient on different study visits. There was, however, a trend towards a small difference in SBF between study visits which emphasizes the importance of vehicle control when investigating the effects of vasoactive compounds. The reasons for this between-day variability are not clear, as ambient temperature was controlled, and subjects were fasted and studied under similar conditions during each study visit. Although careful attempts were made to keep room temperature and conditions constant, the skin is more sensitive than other vascular beds to changes in ambient conditions, and small temperature changes, draughts, or emotional factors may be more important than in other vascular beds. Nevertheless, these between-day differences did

not reach statistical significance and the within-subject and between-day coefficients of reproducibility were similar (3.54 versus 3.48). We conclude that, despite only a small difference between days, vehicle injections should be performed as controls during each study visit.

In conclusion, the technique of intra-dermal drug delivery with saline vehicle coupled with laser Doppler flowmetry to measure skin microcirculatory blood flow offers a safe, well-tolerated, repeatable technique for the investigation of vasoactive compounds in human *in vivo*.

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